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THYMINE INCORPORATION AND
METABOLISM IN ESCHERICHIA COLI

by



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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Thymine Incorporation and Metabolism in Escherichia coli" submitted by Richard Swanson, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The pathway of thymine incorporation into DNA has been examined in several strains of Escherichia coli, including novel temperature-sensitive mutants. The limited utilization of thymine by wild-type cells does not appear to be solely the result of permeability restrictions, nor can it be ascribed to degradation of thymine by these cells.

The effect of mitomycin C on thymineless mutants was studied with respect to intracellular pools of thymine metabolites. It was found that this drug produces an increase in the acid-soluble pool of radioactivity when cells are grown on 2-¹⁴C-thymine. Chromatography of acid-soluble material revealed the presence of dTMP, dTDP, dTTP, thymine and two unidentified compounds. Thymidine was not detected in the acid-soluble pool. In addition mitomycin C induced the development of temperate phages in the two strains of E. coli examined here.

Studies with cell-free extracts prepared from mutant strains have shown that these extracts are able to catalyze the conversion of 2-¹⁴C-thymine to a radioactive compound which has been identified by paper chromatography as thymidylic acid.

The formation of thymidylate was dependent on the presence of a deoxynucleoside 5'-phosphate of which dCMP was the most effective. The rate of formation of thymidylate from 2-¹⁴C-thymine and dCMP was stimulated by addition of various deoxynucleoside mono and triphosphates. The pH optimum for thymidylate formation was between pH 6.5 and pH 7.

The ability of cell-extracts from mutant and wild-type cells to form thymidylate was compared. The extracts from mutant cells catalyzed the formation of thymine to thymidylate at a six-fold greater rate than those from wild-type cells.

Thymineless mutants were isolated by the aminopterin method and were found to require thymine at a much higher level than E. coli strains 5275 or 15 TAU⁻. Two of these mutants gained the ability to incorporate exogenous thymine simultaneously with the loss of ability to synthesize thymidylate.

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ABBREVIATIONS

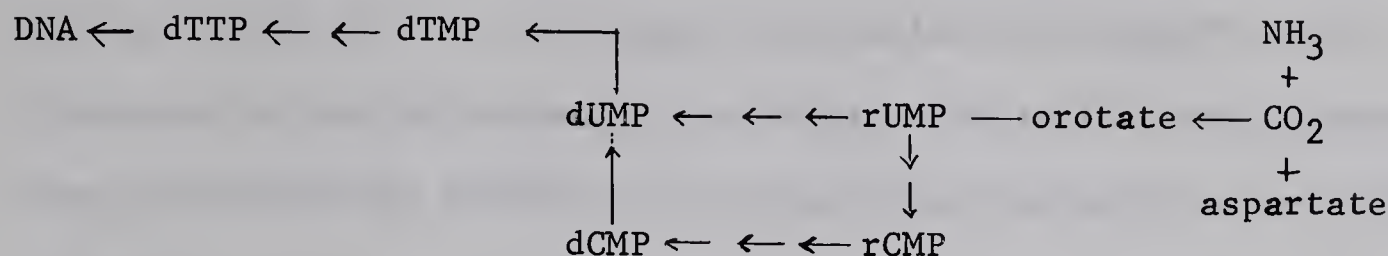
DNA	- deoxyribonucleic acid
MC	- mitomycin C
O.D.	- optical density
EDTA	- ethylenediamine tetra-acetate
Tris	- tris (hydroxymethyl) amino-methane
dCMP	- deoxycytidine 5'-phosphate
dTMP	- deoxythymidine 5'-phosphate
TdR	- thymidine
T	- thymine
PP-ribose-P	- 5-phosphoribosyl 1- α -pyrophosphate

INTRODUCTION

Mutations in a gene controlling the biosynthesis of thymidylate synthetase produce strains of bacteria requiring thymine (Cohen and Barner, 1954). This has been shown in Escherichia coli (Cohen and Barner, 1954), Salmonella typhimurium (Okada et al., 1962), Aerobacter aerogenes (Harrison, 1965), and Bacillus subtilis (Wilson et al., 1966). Extracts of the thymineless (thy⁻) cells are unable to form thymidylate in the presence of deoxyuridylate, formaldehyde, and tetrahydrofolic acid, a reaction sequence which readily occurs in extracts of wild-type cells (Barner and Cohen, 1959; Breitman and Bradford, 1966). In contrast to the prototrophs the mutant cells are capable of incorporating exogenous thymine into DNA (Crawford, 1958): ¹⁴C-labelled thymine is incorporated into the thymidylate residues of DNA (Friesen and Maaloe, 1965). Thymidine, on the other hand, is utilized for DNA synthesis efficiently by both mutant and wild-type cells of E. coli (Boyce and Setlow, 1962) and B. subtilis (Bodmer and Grether, 1965). However, the total amount of thymidine incorporated into the DNA (acid insoluble fraction) of the wild-type cell proved to be very small and decreased rapidly with time. This observation led Rachmeller et al. (1961) to examine the fate of ³H-thymidine in growing cultures of E. coli. They found that when uptake of radioactivity ceased, all the thymidine in the medium had been converted to thymine. The enzyme responsible for this conversion is thymidine phosphorylase. It phosphorytically cleaves thymidine forming thymine and deoxyribose-1-phosphate (Friedkin and Roberts, 1954; Razzell and Khorana, 1958). This enzyme also catalyzes the transfer of deoxyribose from deoxyuridine or deoxythymidine to the pyrimidines thymine or uracil

(MacNutt, 1952; Zimmerman, 1962; Gallo et al., 1967).

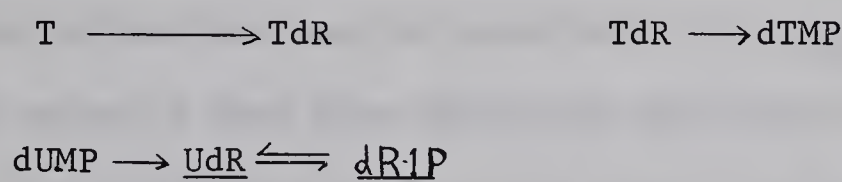
The "de novo" origin of deoxythymidine residues in DNA may be traced through the series of reactions (Magasanik, 1962):



ROUTE I

In this pathway, used by wild-type E. coli, deoxythymidylate is formed by methylation of deoxyuridylate derived from reduction of either uridine diphosphate or cytidine diphosphate (Bertani et al., 1963), through the action of one or more phosphatases. It should be noted that deoxythymidine is never an intermediate.

The auxotroph lacks thymidylate synthetase and consequently cannot convert deoxyuridylate to deoxythymidylate. It is believed that the pathway taken by the auxotroph is as follows:



ROUTE II

In this pathway thymidine phosphorylase transfers a deoxyribose group from either deoxyribose-1-phosphate or deoxyuridine to thymine to form thymidine. This is then phosphorylated by thymidine kinase to thymidylate (Okazaki and Kornberg, 1964), an intermediate in route I. In route II thymidine is an obligate intermediate.

The reason for limited utilization of thymine by wild-type cells is not clear since they possess both thymidine phosphorylase (Razzell and Casshyap, 1964) and thymidine kinase (Okazaki and Kornberg, 1964(a)), and the levels of the two enzymes are similar in the thy⁻ strain. Since thymidine is used efficiently by wild-type cells, it seems apparent that the difference between the strains is at the level of metabolism of thymine.

The problem of defining the mechanism of thymine incorporation in the auxotroph must include a satisfactory explanation for thymine exclusion by the prototroph. Some mechanism must be available to the thy⁻ cell which does not operate in the wild-type cell; that mechanism is not apparent in the reactions shown in route II. The mechanism may be either in the control of known pathways of nucleotide metabolism, or a hitherto undetected reaction. An examination of the possible control sites suggests several alternatives: impermeability of wild-type cells toward thymine, lack of deoxyribosyl donors in wild-type strains, or as the previous sentence has outlined, a new route of thymine metabolism may be operative in the thy⁻ cell. The two enzymes of route II have been mentioned above and do not appear to account for the difference between the mutant and wild-type strains.

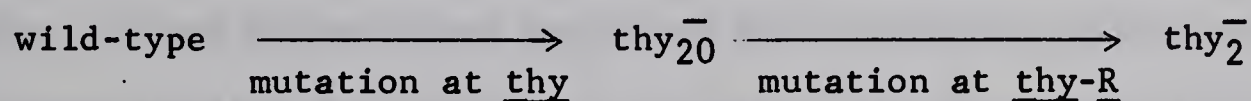
The possibility that the wild-type cell may be impermeable to thymine may be eliminated on the basis of the following experiments. Boyce and Setlow (1962) as well as Budman and Pardee (1967) have shown that high concentrations of deoxyadenosine (1mM) allow wild-type cells to incorporate thymine. Kammen (1967) demonstrated that when wild-type cells were exposed to EDTA they still did not incorporate labelled thymine. That this treatment did indeed relax perme-

ability restrictions was shown by development of sensitivity to actinomycin D after EDTA treatment. Normally E. coli is resistant to this drug, because it cannot enter the cell (Leive, 1965). Prior to the work of Kammen (1967), Swanson and Razzell (1966) have shown that spheroplasts of wild-type E. coli produced by the EDTA-lysozyme treatment (Repaske, 1958) were still unable to incorporate labelled thymine. Spheroplasts of thy⁻ cells incorporated considerable radioactivity into the acid insoluble fraction. Before examining the other various possible sites of control, several observations on types of thymineless mutants of E. coli should be mentioned.

Unlike other auxotrophs which stop dividing in the absence of a necessary growth factor, thymineless mutants die when deprived of thymine (Cohen and Barner, 1954; Cohen and Barner, 1956). The mechanism of thymineless death is not yet clear. Numerous investigations have shown that death in the absence of thymine is due to disturbances in normal DNA replication (Maaloe and Hanawalt, 1961; Friesen and Maaloe, 1965) which may result in: colicin induction (Ishibashi and Hirota, 1965), single-stranded nucleolytic scissions in DNA (Mennigman and Szybalski, 1962), or prophage induction (Endo et al., 1965; Rolfe, 1967).

The phenomenon of thymineless death in bacteria prevents the isolation of thy⁻ auxotrophs by the usual technique. It was found, however, that numerous thymineless mutants of E. coli, Salmonella, and other bacteria can be obtained by the aminopterin method of Okada et al. (1961; 1962). Bertino and Stacey (1966) have suggested the following mechanism for the action of aminopterin in selecting thymineless mutants: thymidylate synthetase (route I) oxidizes

tetrahydrofolate (THFA) during methylation of deoxyuridylate; all other enzymes which transfer one carbon groups do not. Aminopterin inhibits reduction of dihydrofolate (DHFA) to THFA. Thus THFA is consumed in substrate amounts during deoxythymidylate synthesis in wild-type cells, while thy⁻ cells conserve this compound for other synthetic reactions and therefore are selected. Most of the mutants, however, in order to sustain normal growth require 20 times the concentration of thymine required by E. coli 15T⁻ (Cohen and Barner, 1954), a strain used by most investigators before the aminopterin method was discovered. This observation has been reported by a number of workers (Breitman and Bradford, 1964; Harrison, 1965; Okada, 1966). The high thymine requirers seem to occur as a result of a single mutation. Strains with a low thymine requirement can be isolated from these auxotrophs by using a thymine gradient technique (Harrison, 1965). The low thymine requirers are derived from the high requirers as a result of a second mutation in a gene designated as thy-R. This may be represented as:



The first mutational event occurs in the gene coding for thymidylate synthetase. The second mutational event occurs in a second gene which has been located on the chromosome of E. coli K-12 (Okada, 1966). It is far distant from the thymidylate synthetase structural gene. The protein coded for by this gene is not known.

The high thymine requirers, genotype thy⁻ thy-R⁺, resemble the parent wild-type strain more closely than do the low thymine requirers,

genotype thy⁻ thy-R⁻, in that they take up thymidine at low concentrations (1μg/ml), whereas thymine must be present at 10-20 times this concentration for incorporation to occur (Alikhanian et al., 1966). Mutation in the thy -R gene allows the cell to use thymine much more efficiently. It is interesting that some revertants to thymine independence from low thymine requiring strains of E. coli such as 15T⁻ retain the ability to incorporate exogenous thymine (Crawford, 1968). Revertants from "high thymine" strains do not yield prototrophs capable of utilizing exogenous thymine (Harrison, 1965). Harrison (1965) has suggested that the high thymine requirement of some strains was due to an "endogenous inhibitor," because he observed that the growth of these organisms on thymine was inhibited by exogenous cytidine or uridine. Conversely, growth was enhanced by exogenous deoxycytidine or deoxyuridine. He interpreted his results in terms of his hypothetical endogenous inhibitor whose level increases in the presence of cytidine or uridine and decreases in the presence of deoxyuridine, deoxycytidine, or high concentrations of thymine. No experimental evidence for the presence of this inhibitor was presented, however. Other workers have explained this inhibitory effect of the ribosides cytidine and uridine in two ways: (a) formation of thymine riboside, and (b) inhibition of thymidine phosphorylase. In respect to the first, Mantsavinos and Zamenhof (1961) have demonstrated with cell-free extracts of E. coli that uridine greatly stimulates formation of thymine riboside. Cohen and Barner (1956) have shown that this compound inhibits the growth of E. coli. In respect to the second, Budman and Pardee (1967) have found that uridine in vitro inhibits thymidine phosphorylase, an enzyme which they believe is essential in

the conversion of thymine to thymidylate. Their work is open to some question, however, since they assayed the enzyme in the direction of breakdown of thymidine which does not provide information on the direction of synthesis. It would appear at this time that no-one has yet provided a clear-cut mechanism by which ribosides inhibit the growth of thy⁻ cells.

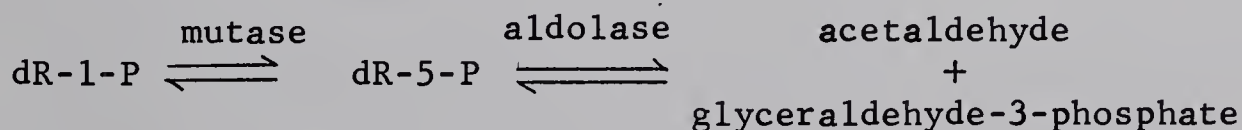
At this point we are left with two important questions, namely; what is the mechanism in E. coli which allows cells lacking thymidylate synthetase to incorporate exogenous thymine, and what is the nature of the mutation which allows thy-R⁻ cells to incorporate thymine more efficiently than their single mutant parents?

The most popular explanation for the fact that wild-type cells do not incorporate thymine is that they lack a source of deoxyribosyl donors such as deoxyuridine, deoxyadenosine, deoxyguanosine, and deoxycytidine. This is based on the fact that thymidine uptake is greatly increased in the presence of deoxyadenosine. The incorporation of thymidine is aided by deoxyadenosine (Boyce and Setlow, 1962) which can provide deoxyribose (Mans and Koch, 1960) to reverse the action of thymidine phosphorylase. The same mechanism allows wild-type cells to incorporate thymine if high concentrations of deoxyadenosine are present in the medium (Budman and Pardee, 1967). These workers also found that thymidine phosphorylase-negative mutants are unable to utilize thymine even if deoxyadenosine is present. They feel that this observation strongly favors thymidine phosphorylase as a key enzyme in the pathway of thymine uptake (route II).

Increased levels of deoxyribosyl donors in mutant as opposed to wild-type cells has been used to explain the differences in uptake

of thymine (Breitman and Bradford, 1964; Kammen, 1967). To support this conclusion these workers cite observations on levels of deoxynucleotides during thymine starvation, and indeed, the levels are significantly elevated over those obtaining during normal growth or in wild-type cells (Neuhard, 1966; Neuhard and Munch-Peterson, 1966). However, if one examines these data it can be seen that during growth in the presence of thymine or, to state the case more clearly, under conditions which must be operative when the cells are incorporating thymine, levels of deoxyribosyl donors are the same as or lower than those in the wild-type cell. Although the possibility of an increased level of some deoxyribose compound not yet considered cannot be overlooked, it would appear that the data at this time are not conclusive.

The significance of the thy-R⁻ mutants in the overall picture of thymine metabolism is not yet clear, nor is the nature of the mutation which accounts for their increased efficiency of incorporation of thymine. Recently, Breitman and Bradford (1967) suggested that the thy-R⁻ mutants are missing the enzyme deoxyribose-5-phosphate aldolase. This enzyme is responsible for degrading deoxyribose-5-phosphate to acetaldehyde and glyceraldehyde-3-phosphate (Pricer and Horecker, 1960). The overall pathway for deoxyribose-1-phosphate degradation is:



The loss of aldolase would prevent such cells from degrading deoxyribose phosphates thus enabling more efficient thymine incorporation. This explanation, however, is not true in all strains since E. coli 5275, another thy-R⁻ strain, possesses normal levels of deoxyribo-

aldolase (Swanson and Razzell, 1966, unpublished observations), as do several strains of thy-R⁻ mutants of S. typhimurium derived by Hoffee (1968). Levels of the mutase in these strains of E. coli have not been described.

The purpose of this investigation is to try to define the factors which account for the difference in thymine incorporation between wild-type and mutant cells.

MATERIALS AND METHODS

1. Materials

All chemicals were obtained from commercial sources and were of a high standard of purity. ^{14}C -thymine was obtained from New England Nuclear Corp., Boston, Mass. and the Radiochemical Centre, Amersham, England. Radiophosphorus ($\text{H}_3^{32}\text{PO}_4$ in dilute HCl) was obtained from Atomic Energy of Canada Limited, Chalk River, Ontario. Before use this was diluted with carrier phosphoric acid and water and evaporated to dryness in vacuo, 2 ml water was added and the evaporation repeated. This procedure removed the HCl . The pyridine used for synthesis of phosphate esters was dried over calcium hydride prior to use. ^{32}P -dCMP was synthesized as described in the methods section. Cyanoethyl alcohol and dicyclohexylcarbodiimide (DCC) were gifts of Dr. A. Hampton, Cancer Research Laboratory, University of Alberta. Aminopterin and mitomycin-C were obtained from Sigma.

Snake venom 5'-nucleotidase was obtained from Calbiochem. Bacterial alkaline phosphatase was obtained from Worthington Biochemicals Corp.

2. Bacterial Strains and their Maintenance

Escherichia coli strain 5275 was obtained from Dr. J. Lederberg, strain 15 TAU⁻ from Dr. P. Hanawalt, strain B from Dr. S. Luria, and strains K12SH and K12SH-28 from Dr. W. Fangman.

Stock cultures were maintained in deeps of half-strength nutrient broth (Difco) plus 1.5% agar, stoppered with corks boiled in paraffin wax, and stored at room temperature. They were kept as long as two years without subculture.

3. Media Employed

(a) Synthetic Medium

The minimal medium employed was slightly modified from that described by B. D. Davis (1950) and contains per 1000 ml:

KH_2PO_4	2.0 g	The glucose and MgSO_4 were autoclaved together, separate from the other salts to avoid caramelization.
K_2HPO_4	7.0 g	
NH_4SO_4	1.0 g	
NaCl	1.0 g	
MgSO_4	0.10 g	
glucose	4.0 g	
arginine	60.0 mg	

pH 7.2

When supplements such as thymine or uracil were used these compounds were added to the medium before sterilization.

(b) Complex Medium

The medium described by Okazaki and Kornberg (1964) was employed and contained in 1000 ml:

glucose	10.0 g
K_2HPO_4	22.0 g
KH_2PO_4	17.0 g
yeast extract (Difco)	10.0 g
thymine	4.0 mg

pH 7.3

Solid media were obtained by the addition of 1.5% agar (Difco) to the preceding. Media were sterilized by autoclaving at 121°C for 15 minutes.

(c) Nutrient Agar

Nutrient agar (Difco) was used for all routine determinations of bacterial numbers.

4. Growth of Organisms

(a) Enzyme Assays

When large quantities of cells were required, bacteria were grown with aeration in 4-8 liter batches in a fermenter (New Brunswick Scientific) at 37°C. Growth was followed turbidimetrically at 650 mμ. Cells were harvested in late log phase, when the optical density was about 2.5 (synthetic medium) or 5.5 (complex medium).

(b) Incorporation Studies

To follow incorporation of radioactive tracers, cells were grown in 16 mm test tubes in a 37°C water bath. Unless otherwise noted, cultures were aerated by bubbling moist air through the fluid. Overnight cultures were grown from stock cultures in a tube roller at 37°C.

5. Preparation of Cell Extracts

Cells were harvested by centrifugation, washed once with 0.05 M Tris malate buffer pH 6.8 containing 0.005 M dithiothreitol and suspended in the buffer to a concentration of 0.25 g wet-weight per ml. The cell suspension, in a glass beaker, was immersed in an ice bath and subjected to sonication for 5 minutes (for 30 ml quantities) using a Bronwill Biosonik (Bronwill Scientific, Rochester, N. Y.) at maximum power output. The temperature of the cell suspension was not allowed to rise above 10°C during this operation. Unbroken cells and debris were removed by centrifugation at 30,000 x g for 10 minutes at 4°C.

The protein content of the cell-free enzyme extracts was estimated by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

6. ^{14}C Thymine Incorporation

To determine the amount of radioactivity entering the acid precipitable fraction of growing cells, aliquots of the culture were removed into an approximately equal volume of cold 0.7 N perchloric acid in a small filter apparatus (Tracerlab) holding a 0.45 μ membrane filter (Millipore Corp.). After 5 minutes contact with the acid, suction was applied and the filter was rinsed several times with 10^{-3} N HCl. The filter was then dried and glued to a planchet. Radioactivity was determined with a Geiger-Müller tube with a thin mica end window (Nuclear Chicago Model 1042). Total radioactivity in cells was determined as above, except that the cells were washed with warm medium instead of acid to remove external thymine. Pool material for study was obtained by the following method. Cells were harvested by centrifugation and washed once with warm medium then the pellet was resuspended in cold 0.4 N perchloric acid. After 10 minutes the suspended cells were removed by centrifugation, so that the supernatant contained the acid soluble material. Nucleotides, bases, and nucleosides were further purified for chromatography by charcoal adsorption. Compounds were eluted from the charcoal by three successive washings with 50% ethanol containing 1% concentrated ammonium hydroxide. The eluate was concentrated "in vacuo".

7. Isolation of Mutants

Thymine-less auxotrophs were acquired by incubating cells (initial cell density 2×10^8 per ml) for 48 hours at 37°C in liquid mineral salts medium supplemented with 300 $\mu\text{g/ml}$ aminopterin and 50 $\mu\text{g/ml}$ thymine (Stacey and Simpson, 1965). Dilutions of these cultures were plated on mineral salts agar supplemented with 50 $\mu\text{g/ml}$ thymine followed by replica-plating (Lederberg and Lederberg, 1952). Thymine-less bacteria

obtained in this way required 20 - 25 $\mu\text{g/ml}$ thymine to sustain growth. Two of the mutants isolated from strain B could grow at 25°C in the absence of thymine but not at 37°C. These latter strains required 20 $\mu\text{g/ml}$ thymine for growth at the non-permissive temperature. Strains with a low thymine requirement were isolated from the temperature-sensitive strains as follows. Approximately 10^9 washed cells were spread onto a plate of minimal agar, 1 mg of dry thymine was placed in the center, and the plate was then incubated at 37°C. After 2 days incubation, satellite colonies appeared at the periphery of the confluent growth surrounding the thymine. These colonies were picked, tested for their ability to grow on 2 $\mu\text{g/ml}$ thymine, and stock cultures were prepared.

8. Preparation of Spheroplasts

Spheroplasts of E. coli were prepared by a method similar to that described by Repaske (1958). Exponential cultures of strains B and 5275 in minimal medium supplemented with 4 $\mu\text{g/ml}$ thymine were harvested by centrifugation, washed once with warm medium and suspended in minimal medium containing 20% sucrose, 10^{-3} M EDTA and 10 $\mu\text{g/ml}$ lysozyme. After 5 minutes at 30°C aliquots of the suspensions were diluted fourfold with water to determine osmotic fragility (decrease in O.D. at 650 m μ). The protoplast suspensions were then centrifuged at 2000 x g for 10 minutes, and the pellets were suspended in prewarmed minimal medium containing 20% sucrose and 1 $\mu\text{g/ml}$ ^{14}C -thymine (60,000 c.p.m./ml). Thymine incorporation was determined as described on page 13.

9. Chromatography

(a) Paper Chromatography

Paper chromatography was carried out at room temperature by the

descending technique using Whatman No. 40 or 3 MM paper. The solvent systems used were: Solvent A, isobutyric acid-ammonium hydroxide-water-0.1 M EDTA (100:4:56:2); Solvent b, ethyl propionate-formic acid-water (65:5:35) the upper organic phase was used; Solvent C, isopropanol-conc. ammonium hydroxide-water (7:1:2); Solvent D consisted of 60 parts of a 1 M ammonium acetate solution saturated with sodium tetraborate with 0.01 M EDTA pH 9 and 140 parts of 90% ethanol. The ultraviolet absorbing material was observed with short wave uv light (Mineralight C-51). Radioactivity on the chromatograms was detected using a Nuclear Chicago Actigraph III.

The following technique was employed for elution of compounds from chromatograms. A strip containing the desired compound was cut from the dried chromatogram sheet, one end of the strip dipped in the eluent and the fluid allowed to rise up the paper. The strip was then wrapped in aluminum foil for support and placed in a centrifuge tube with the end distal to the eluent reservoir entering the tube first. After the upper end of the paper-aluminum foil strip was fastened over the lip of the centrifuge tube with a rubber band, the solvent was spun off by centrifuging at slow speed.

(b) Thin Layer Chromatography

The chamber used for thin layer chromatography was a product of Eastman Kodak Co. (Eastman Chromagram Chamber Plate Set). MN polygram sheets of either cellulose (CEL uv 254) or silica gel (SIL S/uv 254) were employed using the standard ascending technique. The sheets contained a non-soluble fluorescent dye for detection of ultraviolet absorbing spots.

10. Synthesis of dCM³²P

dCM³²P was synthesized by published methods (Smith and Khorana, 1963), except dCMP itself was used as a starting material, the phosphate group blocking the 5' position during acetylation. This was subsequently removed by incubation of the N⁶-3'-O-diacetyldeoxycytidine-5' phosphate with bacterial alkaline phosphatase at pH 8. The product was phosphorylated with previously synthesized cyanoethyl phosphate-³²P. After work up of the reaction mixture and chromatography on a small column of Dowex 1X8 formate, the desired peak after concentration was further purified by chromatography in solvent A and eluted from the paper. The eluate was chromatographically pure and behaved identically with a sample of deoxycytidine-5' phosphate obtained commercially. Furthermore, it was completely degraded by crude snake venom to deoxycytidine.

11. Measurement of Deoxythymidylate Formation

The rapid technique of Sherman (1963) was used to measure the conversion of ¹⁴C-thymine to dTMP. This assay depends on the formation of negatively charged products from radioactive non-charged substrate. The charged compounds are adsorbed to the DEAE (Whatman DE81) disc while unreacted substrate is eluted. The DEAE discs were then dried and radioactivity determined as described before. The DEAE discs (25 mm in diameter) are held in the millipore filter device mentioned previously and were wetted with water before contact with the assay mixture to prevent non-specific binding of thymine.

EXPERIMENTAL RESULTS

The thymine deficiency of E. coli mutants 14 TAU⁻ and 5275 (strains used predominantly in this investigation) may be alleviated by either thymine or thymidine in low concentrations (ca. 2 μ g/ml). Absence of thymine resulted in no growth in overnight culture. A characteristic feature of thymineless bacteria is that they die when allowed to metabolize in the absence of thymine. Figure 1 shows the effect of thymine starvation on growth (O.D.) and viability of E. coli 5275. During the period of thymine starvation protoplasmic synthesis is evident by the increase in optical density. Microscopically, the cells appear elongated because of the absence of cell division. The lag period before loss of viability occurs is characteristic of thymineless death in E. coli (Freifelder and Maaloe, 1964).

Figure 2 shows growth and ¹⁴C thymine incorporation into acid-insoluble material in strain 5275.* When the cold acid-insoluble material was heated in a steam bath with 0.7N perchloric acid for 30

* In earlier experiments ³H thymine (Merck, Sharp and Dohme) was used, but it was found that label entered wild-type as well as mutant cells. Further study revealed that, of the acid insoluble radioactivity in the cells, 70% was in the protein fraction. Although the ³H thymine did not separate into components in two different solvent systems (B,C) and was all absorbed onto charcoal from dilute acid, it was concluded, on the basis of the bacterial studies that it consisted of a mixture of compounds.

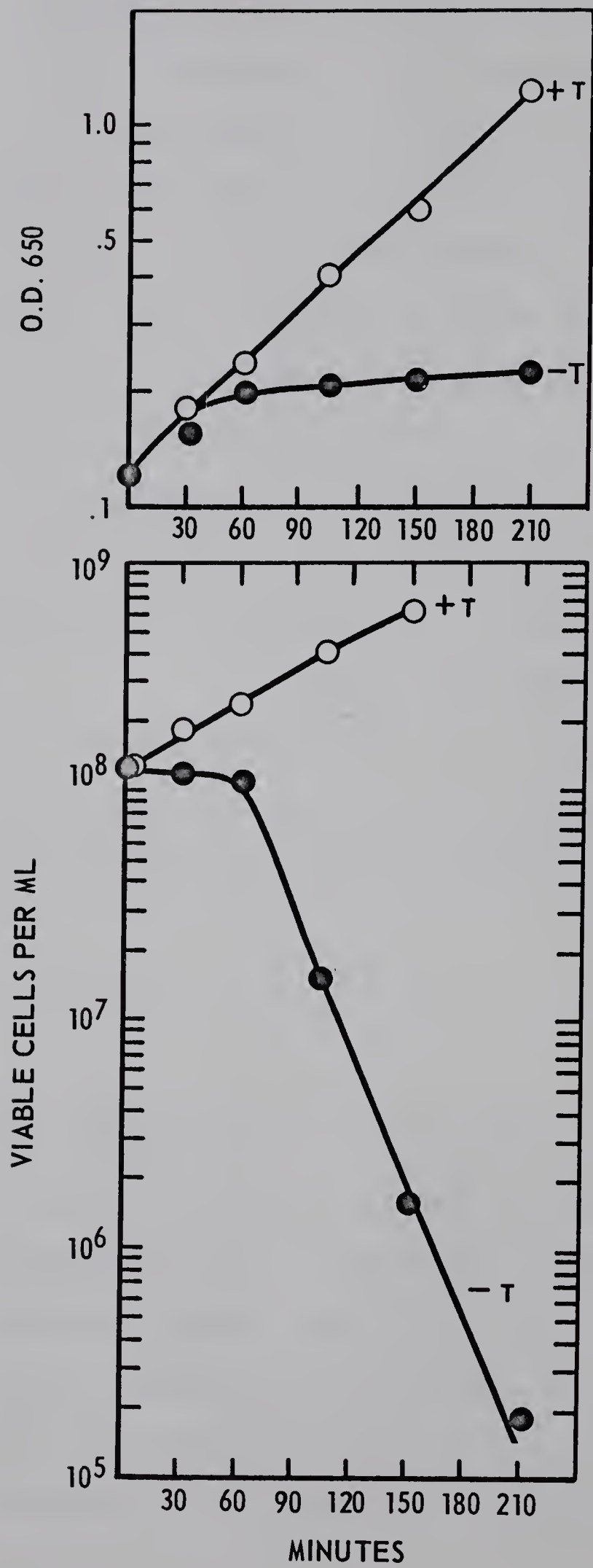


FIGURE 1

THYMINE-LESS DEATH IN E. COLI 5275

Bacteria from an overnight culture in synthetic medium supplemented with 4 $\mu\text{g/ml}$ thymine were harvested by centrifugation, washed once with minimal medium, and resuspended in the same medium to 2×10^8 cells/ml. Thymine was added to one tube at a concentration of 4 $\mu\text{g/ml}$ as a control. Optical density was followed in both tubes and viable count was followed in the thymine-less culture by plating suitable dilutions on nutrient agar plates.

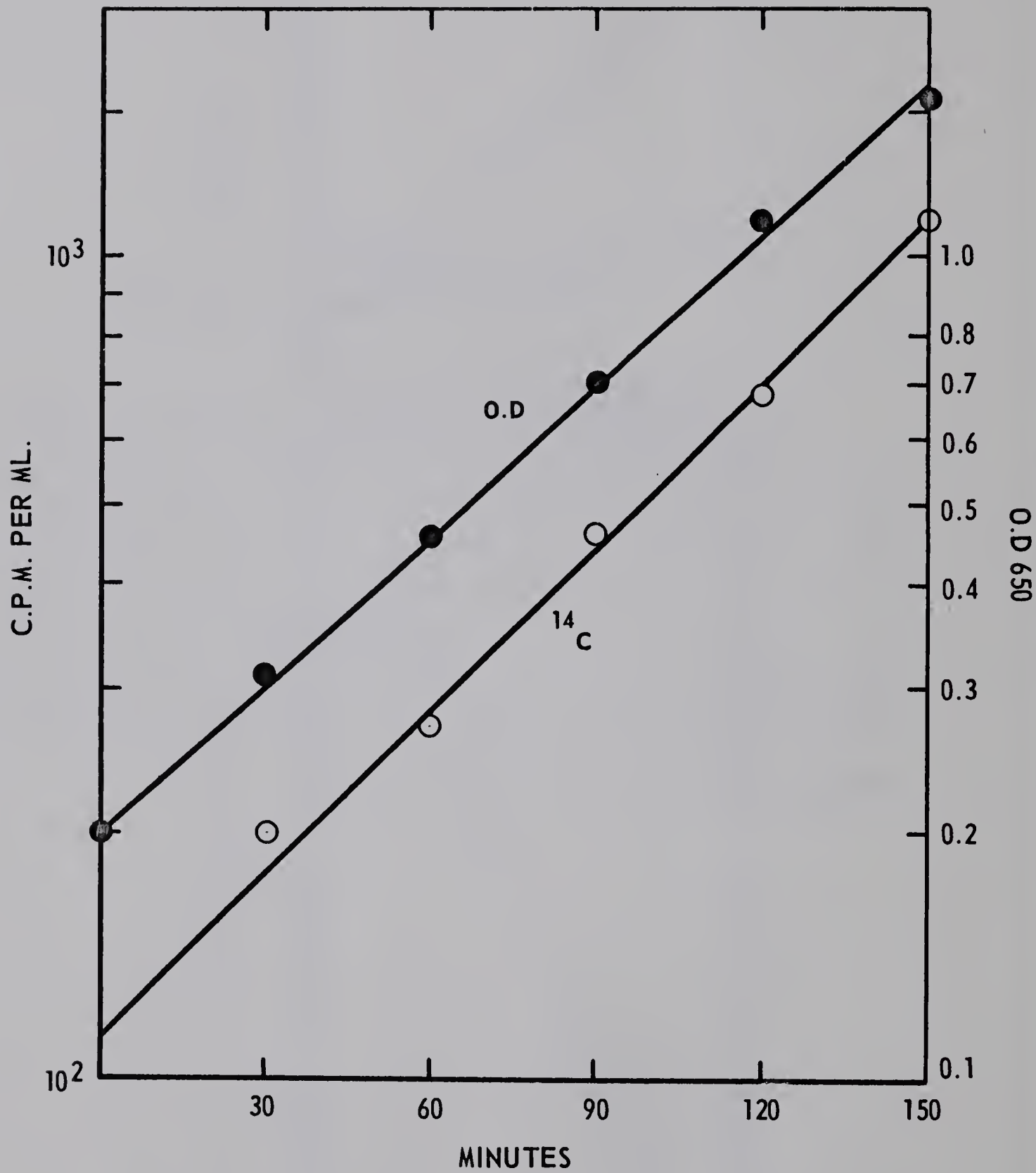


FIGURE 2

GROWTH AND THYMINE INCORPORATION IN E. COLI STRAIN 5275

Growth and acid-insoluble thymine incorporation were determined as described in Materials and Methods. The thymine concentration was 4 μ g (6000 c.p.m.) per ml.

minutes, 91% of the radioactivity was made acid soluble. The pellet contained 1.0% of the radioactivity. This indicates that thymine is entering the nucleic acid fraction.

A comparison of thymine incorporation in wild-type and mutant bacteria is shown in Table I.

TABLE I

Strain	Thymine Incorporated
	μ moles per 10^9 Cells
B <u>thy</u> ⁺	0.26
3000 <u>thy</u> ⁺	0.24
5401 <u>thy</u> ⁺	0.21
5275 <u>thy</u> ⁻	5.6

The wild-type cells incorporated only 5% as much thymine as the mutant cells. It is apparent that a small amount of exogenous thymine is incorporated by the wild-type strains, however this would supply only 1/20 of that required for DNA synthesis in this experiment. The balance must be synthesized "de novo".

Thymine Incorporation in Spheroplasts - If permeability restrictions alone are responsible for the limited utilization of thymine by wild-type cells, a modification of the cell-wall-membrane structure should affect the pattern of thymine uptake. In view of the fact that spheroplasts of E. coli are "permeable" (i.e. susceptible) to bacteriophage DNA (Fraser, et al., 1957), a property not shared by untreated cells, spheroplasts should exhibit a relaxation of permeability barriers. That EDTA-lysozyme treated cells are more permeable may be inferred from

the results of Kammen (1967) (which were unavailable at the time this experiment was performed). He showed that treatment with EDTA alone at similar concentrations induces sensitivity to Actinomycin D (see p. 3). Accordingly, spheroplasts of E. coli B with 5275, as a control, were prepared, and their ability to incorporate ^{14}C thymine determined. The results of this experiment are presented in Figure 3. It is apparent that spheroplasts do not differ in the pattern of thymine uptake from whole cells. This experiment coupled with the work of Kammen (1967) indicates that permeability restrictions alone cannot account for the limited thymine uptake in the wild-type cell.

Thymine Degradation - in mammalian cells thymine is degraded to methyl malonic semialdehyde, the 2 carbon of the ring appears as CO_2 (Canellakis, 1956). Since most of the thymine used in experiments reported so far has been labelled in the 2 carbon, it seemed possible that the wild-type organism degraded thymine at a much greater rate than the mutant; the CO_2 formed would then be lost. To test this hypothesis strains B, 3000, and 5275 were grown in stoppered tubes in the presence of ^{14}C thymine. Air was passed through gently, and the effluent gas was trapped in NaOH. When growth was complete the cells were removed and the culture supernatant was charcoal adsorbed. The radioactivity in the cells, culture medium after charcoal, and the NaOH were determined. The results of this experiment are shown in Table II. Neither the auxotroph nor the wild-type strains appear to degrade thymine, since no appreciable radioactivity appeared in the NaOH. Furthermore, the radioactivity remaining in the supernatant after removal of cells, although not chromatographed, was predominantly charcoal adsorbable, since 99% of the label was removed by charcoal. These findings indicate that degradation of exogenous thymine by wild-type cells does not account for the difference in incorporation.

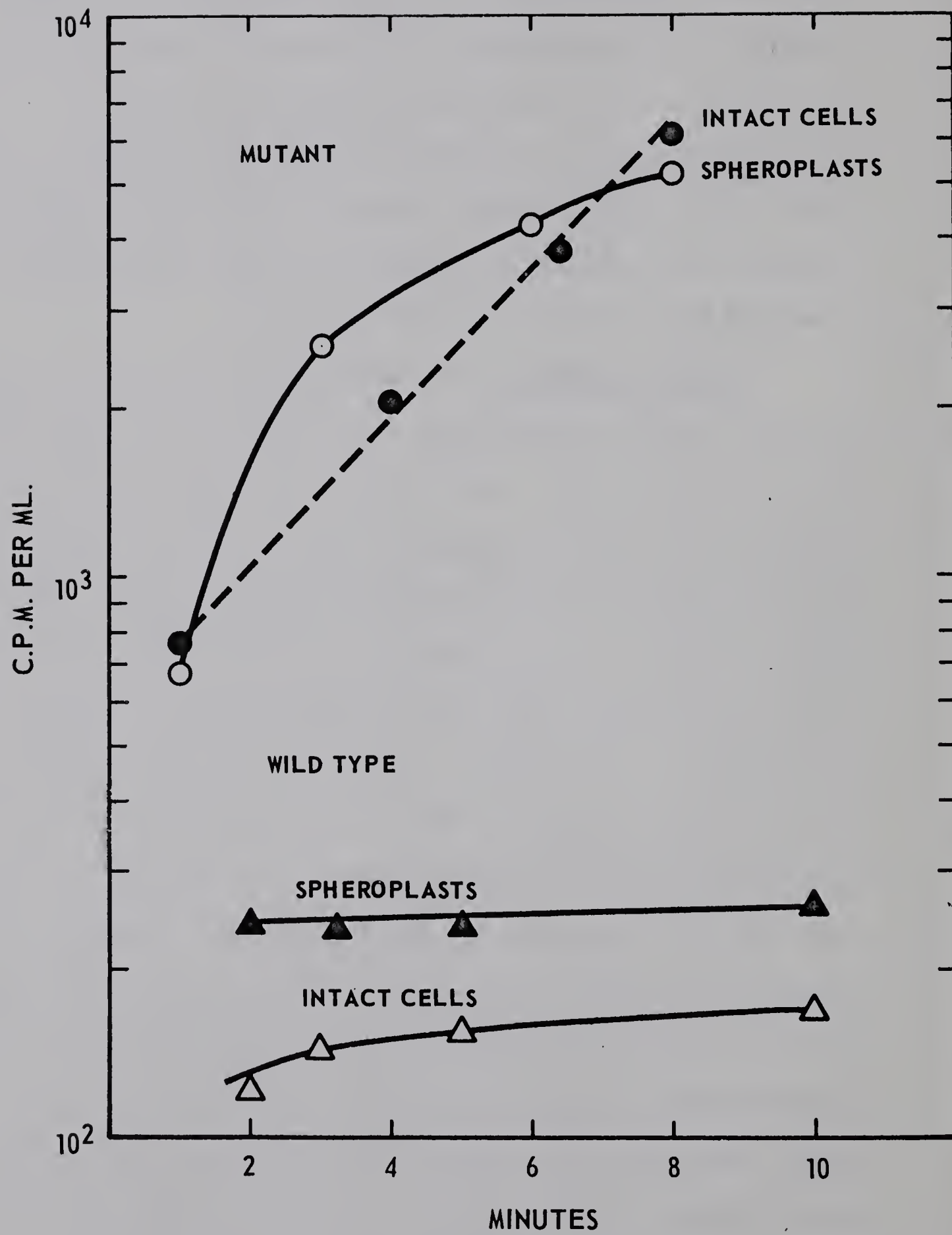


FIGURE 3

THYMINE INCORPORATION INTO SPHEROPLASTS OF E. COLI B AND 5275

Spheroplasts were prepared as described in Materials and Methods. A fourfold dilution of spheroplast suspension led, in each strain, to a 95% decrease in OD at 650 m μ indicating extensive lysis. Uptake of radioactivity into the acid-insoluble fraction was followed as described in Materials and Methods.

TABLE II
FATE OF 2-¹⁴C-THYMINE

Strain	Radioactivity in NaOH	Total Radioactivity in Cells	Radioactivity in Medium	Radioactivity in Medium After Charcoal
	c.p.m.	c.p.m.	c.p.m.	c.p.m.
B	60	600	124,000	1,570
3000	78	400	119,000	1,380
5275	64	54,000	60,500	770

6 ml of minimal medium supplemented with 4 μ g (21,000 c.p.m.)/ml thymine were inoculated with overnight cultures of the indicated strains. The cultures were contained in test tubes stoppered with 2-hole stoppers and were aerated with moist air from which CO₂ had been removed with NaOH. The effluent gas was passed through a column of glass beads moistened with 5N NaOH. When the optical density of the cultures had reached 2.5 they were harvested by centrifugation, and the pellets were dissolved in NaOH. 3 ml of the cell-free medium was then acidified (0.2 ml 60% perchloric acid), and 50 mg of activated charcoal was added. After 5 minutes the charcoal was removed by centrifugation. Aliquots of each fraction were then placed on planchettes and the radioactivity determined.

Intracellular Pools of Thymine Metabolites - As mentioned in the introduction, known enzymatic reactions do not adequately define a mechanism of control of thymine metabolism in auxotrophs. Most of the studies have been carried out with cell-free extracts with little attention being given to sequences of reactions occurring "in vivo". It therefore seemed useful to look at the cellular pools of thymine metabolites during growth of these auxotrophs. It was hoped that conditions could be manipulated so that individual intermediates would accumulate and thus provide information on the whole reaction sequence. In order to examine cellular pools of thymine metabolites which are extremely low under normal growth conditions (Neuhard, 1966), a means of specifically inhibiting DNA synthesis without interfering with the formation of DNA precursors was sought. Many drugs which inhibit DNA synthesis act by blocking the synthesis of deoxynucleotides and therefore could not be used.

The antibiotic mitomycin C isolated from Streptomyces caespitosus (Hata et al., 1960) appeared to be the drug of choice. Its mechanism of action is believed to involve crosslinkage formation in the DNA molecule itself, the crosslinkages (between guanosine and cytosine) thereby prevent replication of the DNA molecule (Dyer and Szybalski, 1963, 1964). Figure 4 shows the effect of 10 µg/ml of mitomycin C on incorporation of ¹⁴C thymine into the acid soluble and insoluble fractions of E. coli 5275. The drug effectively inhibits DNA synthesis but permits an appreciable increase of radioactivity in the acid soluble pool.

Unfortunately, experiments of the above type could not be prolonged because of induction of prophage by mitomycin C. This phenomenon has been extensively reported in the literature for E. coli (Korn and

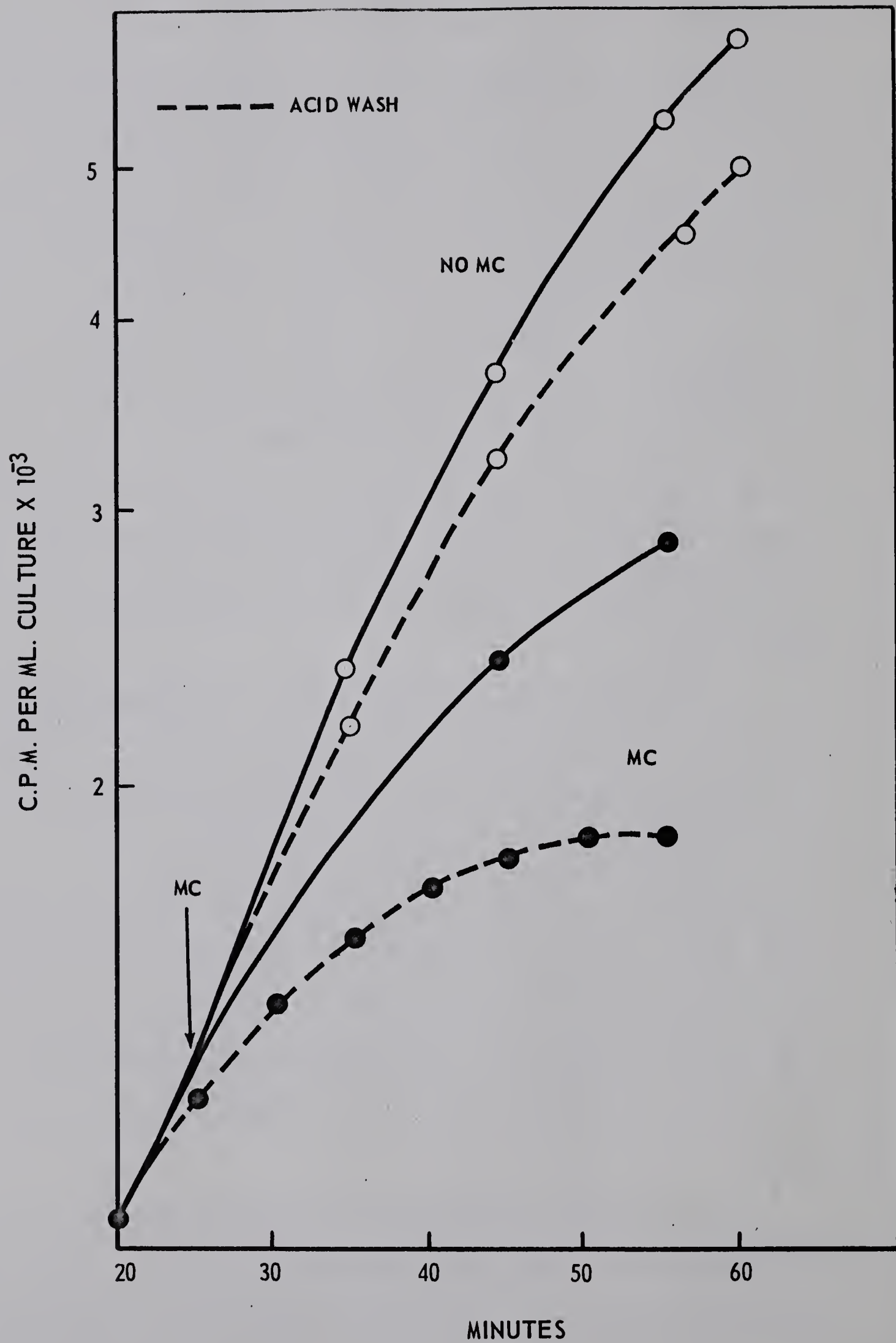


FIGURE 4

EFFECT OF MITOMYCIN C ON THYMINE UPTAKE IN *E. COLI* 5275

E. coli 5275 (6.7×10^8 cells/ml) in minimal medium supplemented with thymine $2\mu\text{g}$ (3×10^5 c.p.m.)/ml were exposed to mitomycin C ($10\mu\text{g/ml}$). Samples of culture were removed and assayed for incorporation of label into cells (total) and into acid-precipitable material. Total radioactivity was determined by rinsing the cells on the millipore filter with warm medium, acid-precipitable radioactivity was determined as described in Materials and Methods. The pool material is represented by the difference between total c.p.m. and acid insoluble c.p.m.

Wēissbach, 1962; Levine, 1961; Otsuji et al., 1959) and for B. subtilis (Seaman et al., 1964). Two thymineless strains of E. coli were used to try and avoid this difficulty; strain 5275 derived from K12 appeared to produce a phage morphologically similar to λ (Figure 5) and strain 15 TAU⁻ gave rise to what appeared to be phage heads, tails, and a complete phage morphologically unrelated to the heads or tails (Figure 6). The effect of mitomycin C (10 μ g/ml) on ¹⁴C thymine incorporation into the acid insoluble fraction of E. coli 15 TAU⁻ is shown in Figure 7. It may be seen that inhibition of DNA synthesis is marginal and that prophage induction occurs very soon after mitomycin C addition (shown by rapid rate of ¹⁴C incorporation 30 min. after addition of mitomycin C). Since mitomycin C had such a marginal effect on strain 15 TAU⁻ strain 5275 was used for the remainder of these experiments.

Figure 8 shows an actigraph scan of a chromatogram (solvent A) of the acid soluble fraction from 5275 after 30 minutes growth in the presence of 10 μ g/ml mitomycin C and ¹⁴C thymine. The compounds indicated were chromatographed in other solvent systems before and after dephosphorylation (see below). The two small peaks between thymine and deoxythymidylate were examined in solvent systems 2 and 3 after elution. Solvent D was used to determine if these compounds possessed cis-hydroxyl groups (these complex with the borate in the system and are thus retarded). Both compounds appear to possess cis-hydroxyl groups. Chromatography of peak A in solvent 3 indicated that the compound was not phosphorylated. This compound may be ribosyl thymine. There was an insufficient quantity of the material in peak B for further study. Although the identity of these unknown thymine compounds could not be determined, chromatography has demonstrated that they are not thymidine. The lack of accumulation of this compound in cells inhibited by mitomy-

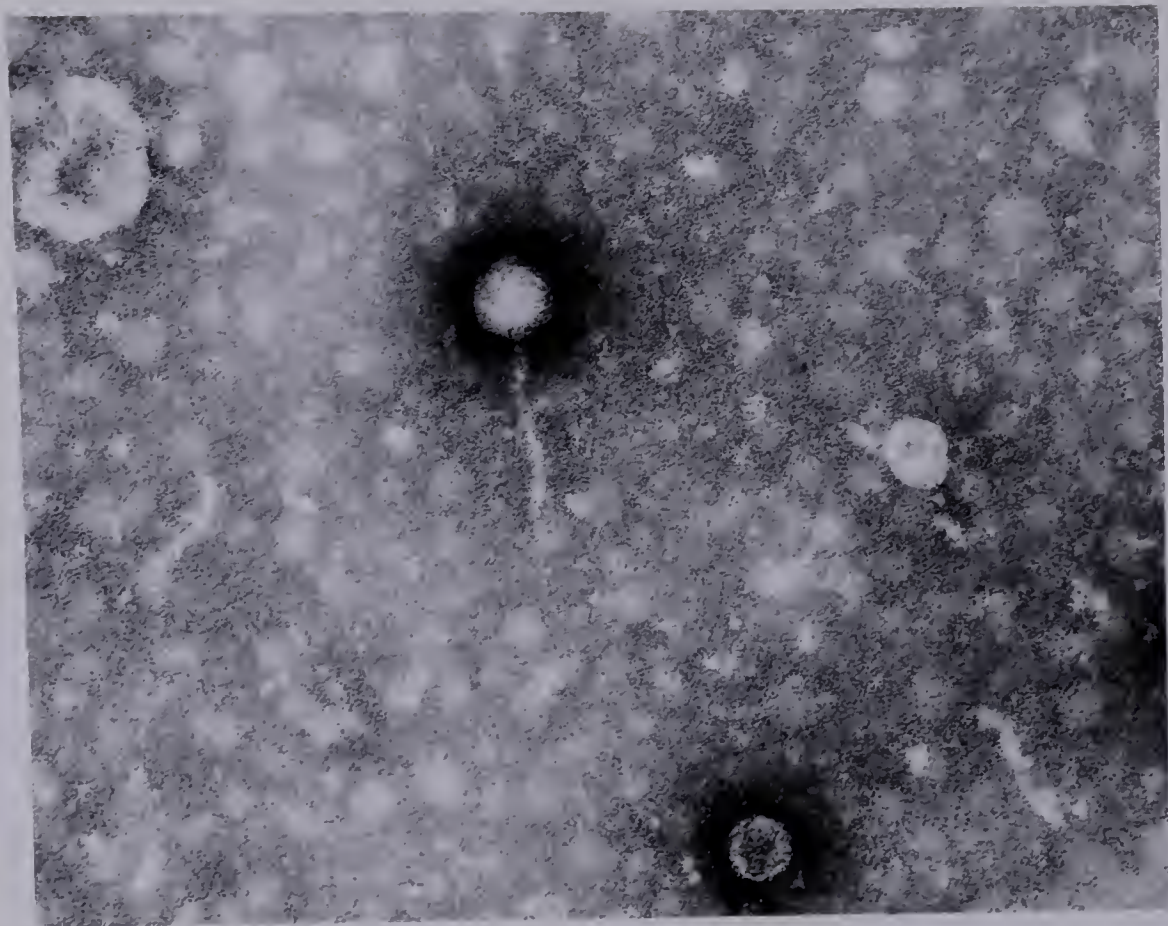


FIGURE 5

PHAGE INDUCED IN E. COLI 5275 BY MITOMYCIN C

A log phase culture in minimal salts medium supplimented with 4 $\mu\text{g/ml}$ thymine was exposed to 10 $\mu\text{g/ml}$ mitomycin C. After 2 hours contact, samples were removed into an equal volume of 3% phospho-tungstic acid solution adjusted to pH 7.0 (Brenner and Horne, 1959). A droplet of this mixture was placed on a formvar coated copper grid, excess fluid removed with filter paper, and air dried. The preparations were then examined in a Phillips 200 Electron Microscope and photographed. X 80,000



FIGURE 6

PHAGE INDUCED IN E. COLI 15 TAU⁻ BY MITOMYCIN C

A log phase culture in minimal salts medium supplemented with 4 µg/ml thymine and 10 µg/ml uracil was exposed to 10 µg/ml mitomycin C. After 160 minutes contact, the cultures were centrifuged at 5000 x 6 for 5 minutes. The supernatant containing the phage was then centrifuged for 3 hours at 45,000 x 6. The pellet was resuspended in 0.01 M Tris acetate pH 7.5. This preparation was prepared and stained as in legend of Figure 5. X 90,000

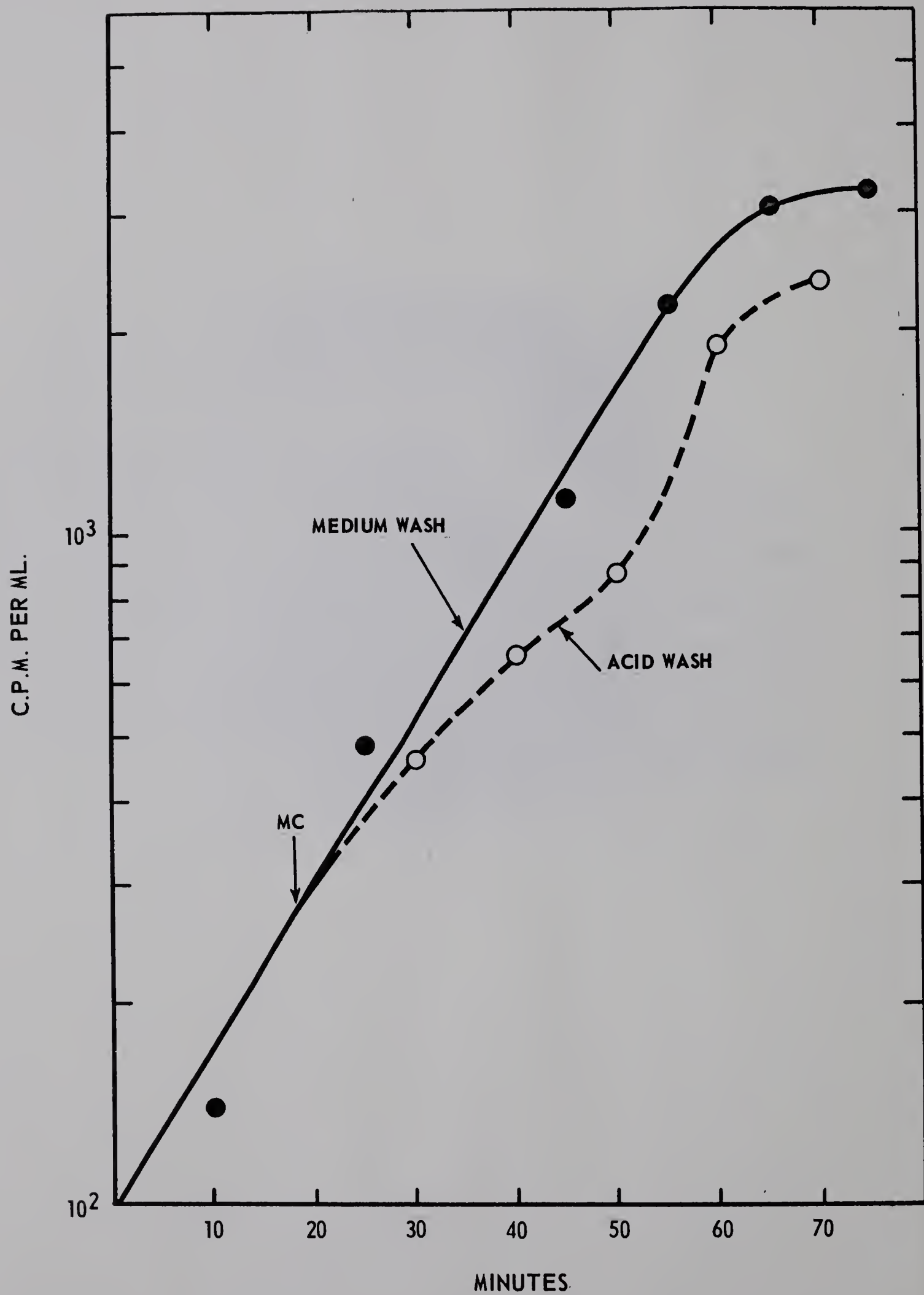


FIGURE 7

EFFECT OF 10 $\mu\text{g}/\text{ml}$ MITOMYCIN C ON E. COLI 15 TAU⁻

For legend see Figure 4.

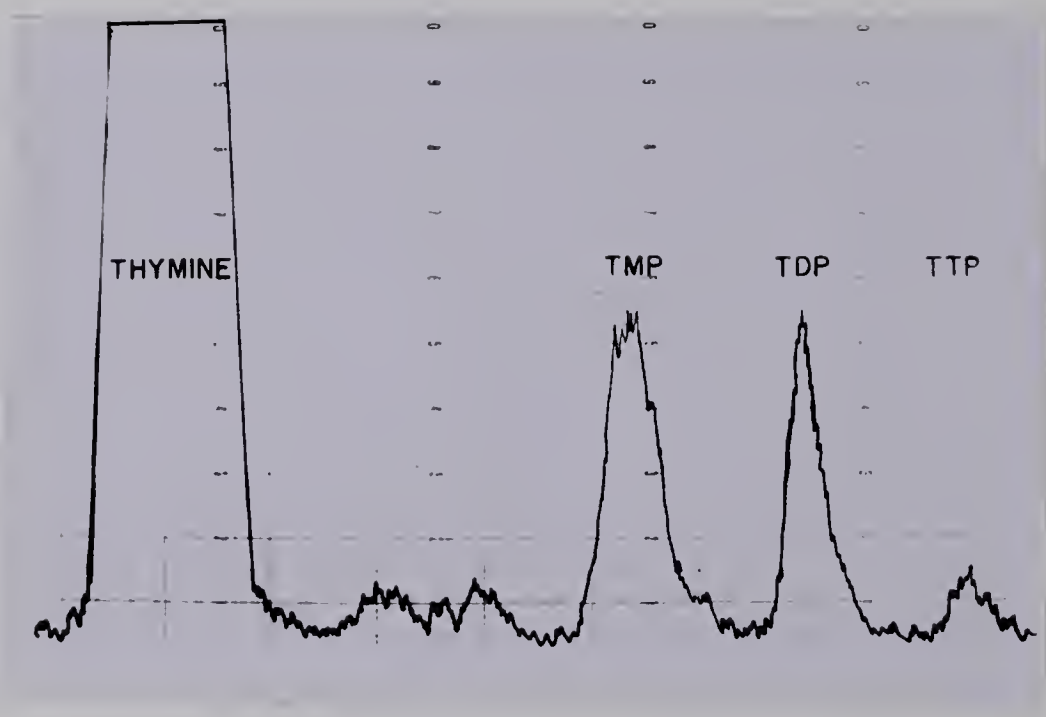


FIGURE 8

CHROMOTOGRAM OF THE ACID-SOLUBLE POOL
OF E. COLI 5275 GROWN IN THE PRESENCE OF
MITOMYCIN C AND ^{14}C THYMINE

Two ml of E. coli 5275 (10^9 cells/ml) were simultaneously exposed to $1\text{ }\mu\text{g}$ ($1.6\text{ }\mu\text{C}$)/ml thymine and $10\text{ }\mu\text{g}/\text{ml}$ mitomycin C. After 30 min. the cells were harvested by centrifugation, washed once with medium and extracted with 2 ml 0.4N perchloric acid. The acid soluble radioactive compounds were adsorbed on charcoal (98% of radioactivity), the charcoal washed once with water and the charcoal eluted with 50% ethonol, 1% ammonium hydroxide. The eluate was then concentrated, and chromatographed in solvent A. The tailing edge of the thymine peak was eluted and chromatographed in solvent B.

cin C was surprising since thymidine is supposed to be an intermediate in thymidylate biosynthesis (route II, page 2); its absence suggests the possibility of an alternate route from thymine to thymidylate, perhaps involving direct conversion of thymine to thymidylate.

Since no thymidine was found in the pool of mitomycin C inhibited cells, the possibility existed that thymidine kinase was present in excess and that the thymidine phosphorylase reaction was rate-limiting. If indeed phosphorylation was removing thymidine from the pool as fast as it could be formed then conceivably one could block this reaction by depleting the cellular pool of ATP, the phosphate donor in this reaction. Pardee (1962) and Kovac and Kuzela (1966) have reported 2,4 dinitrophenol (DNP) effectively inhibits protein synthesis by blocking energy producing reactions. An attempt was made, therefore, to employ DNP in conjunction with mitomycin C in order to block phosphorylation of thymidine if it was formed, thus increasing its concentration in the pool. Several concentrations of DNP were tried (0.2, 0.5, 2.0 mM), however, this compound completely blocked uptake of ^{14}C -thymine into the acid soluble pool, and this approach was abandoned. The blockage of uptake of thymine into the pool of E. coli suggests an energy requirement for thymine uptake; however, more work would be needed to confirm such a requirement.

Isolation of Thymineless Mutants - The isolation of additional thymineless mutants was undertaken in order to obtain mutants requiring high concentrations of thymine for growth. Furthermore, Alikhaninian et, al. (1966) reported that some of the aminopter-in-derived mutants isolated by them were thymine-dependent at 30°C but required thymine for growth at 37°C. Presumably this temperature sensitivity is due to the production of a temperature-sensitive thymidylate synthetase.

Since these mutants could be made thymine-requiring simply by raising the temperature, it was hoped that they would serve as a particularly useful tool for the study of control of thymine uptake.

E. coli K12SH and B were chosen as the parent strains, and the aminopterin method (Materials and Methods) was employed to select thymineless bacteria. In this experiment thirteen mutants were isolated. Eight were derived from strain K12SH and five from strain B. All the mutants isolated required at least 15 $\mu\text{g/ml}$ thymine for growth, but the optimal concentration appeared to be about 25 $\mu\text{g/ml}$. Upon further screening two of the mutants derived from E. coli B (designated B-1 and B-3) were found to be temperature-sensitive, requiring thymine at 37°C but not at 30°C.

Since a characteristic feature of normal thymineless mutants is that they die in the absence of thymine it was of interest to investigate whether thymineless death is characteristic of the temperature-sensitive strains. The results of thymine starvation in strain B-1 are shown in Figure 9. The lag preceding death and, in fact, the entire sequence of events closely follows those seen in the low-thymine requiring strain 5275 (p. 18). Although strain B-1 undergoes thymineless death in the presence of 3 $\mu\text{g/ml}$ thymine, at this concentration the cells become very elongated and continue to synthesize cell material at an appreciable rate for about 4 hours. These filamentous cells may reach lengths of over 100 μ . During this time the optical density increases 10 - 12 fold. In such cultures less than 0.01% of the cells remain viable.

Thymine Uptake in Strain B-1 at Permissive and Non-permissive Temperatures - Since thymine is excluded from the DNA of wild-type

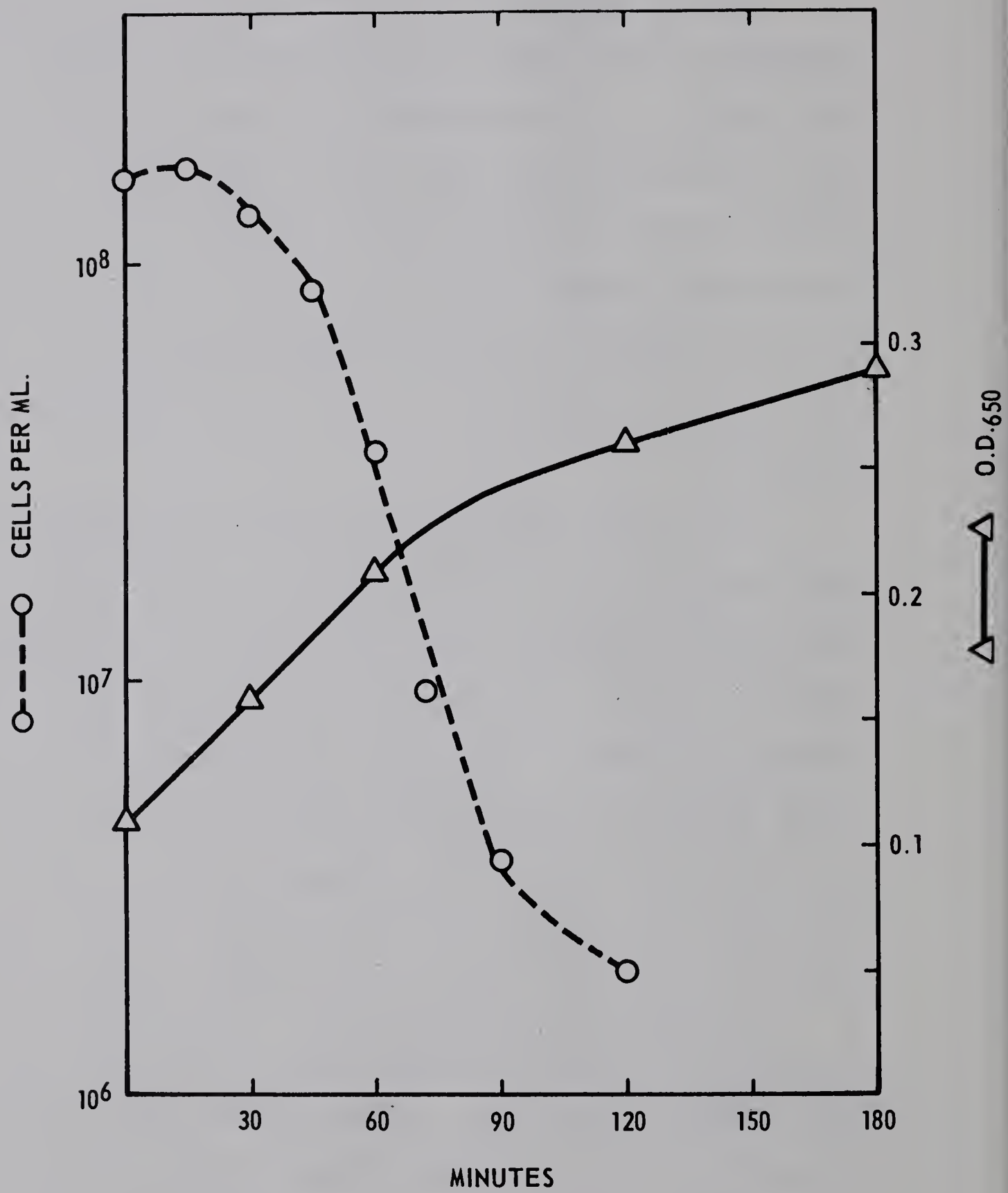


FIGURE 9

THYMINELESS DEATH IN E. COLI B-1

For legend see Figure 1.

organisms it was of interest to examine thymine uptake in the temperature-sensitive strain. Two levels of thymine were used (3 $\mu\text{g}/\text{ml}$, 30 $\mu\text{g}/\text{ml}$), and thymine uptake was followed for 150 minutes before the temperature was increased to 37°C (Figure 10). Very little thymine was incorporated at the permissive temperature, but the rate of incorporation began to increase soon after the temperature was raised. It is interesting that although this strain incorporated an appreciable amount of thymine at the low concentration it nevertheless underwent thymineless death. This may be explained if we assume that the lower rate of thymine incorporation in the presence of 3 $\mu\text{g}/\text{ml}$ results in a subnormal rate of DNA synthesis causing thymineless death.

The incorporation data of Figure 10 indicate that this mutant utilizes external thymine only when its endogenous supply is blocked at the elevated temperature. It seems as though in high thymine requiring mutants, endogenous synthesis of thymidylate and the ability to incorporate thymine are mutually exclusive properties. Although no further work was done with this temperature-sensitive strain, the information provided by this experiment indicates that cells which normally do not incorporate thymine possess the genetic capacity to do so when their "de novo" route is blocked. It is still not known, however, whether the ability to incorporate thymine is due to a change in the enzymatic make-up of the cell through induction or repression or simply an alteration in the level of metabolite(s) (i.e. deoxyribosyl donors or effectors) within the cell.

Several strains of low thymine requiring mutants were isolated from strain B-1, and these also retained the capacity to synthesize their own thymine, requiring this compound only at 37°C. These mutants were not studied further.

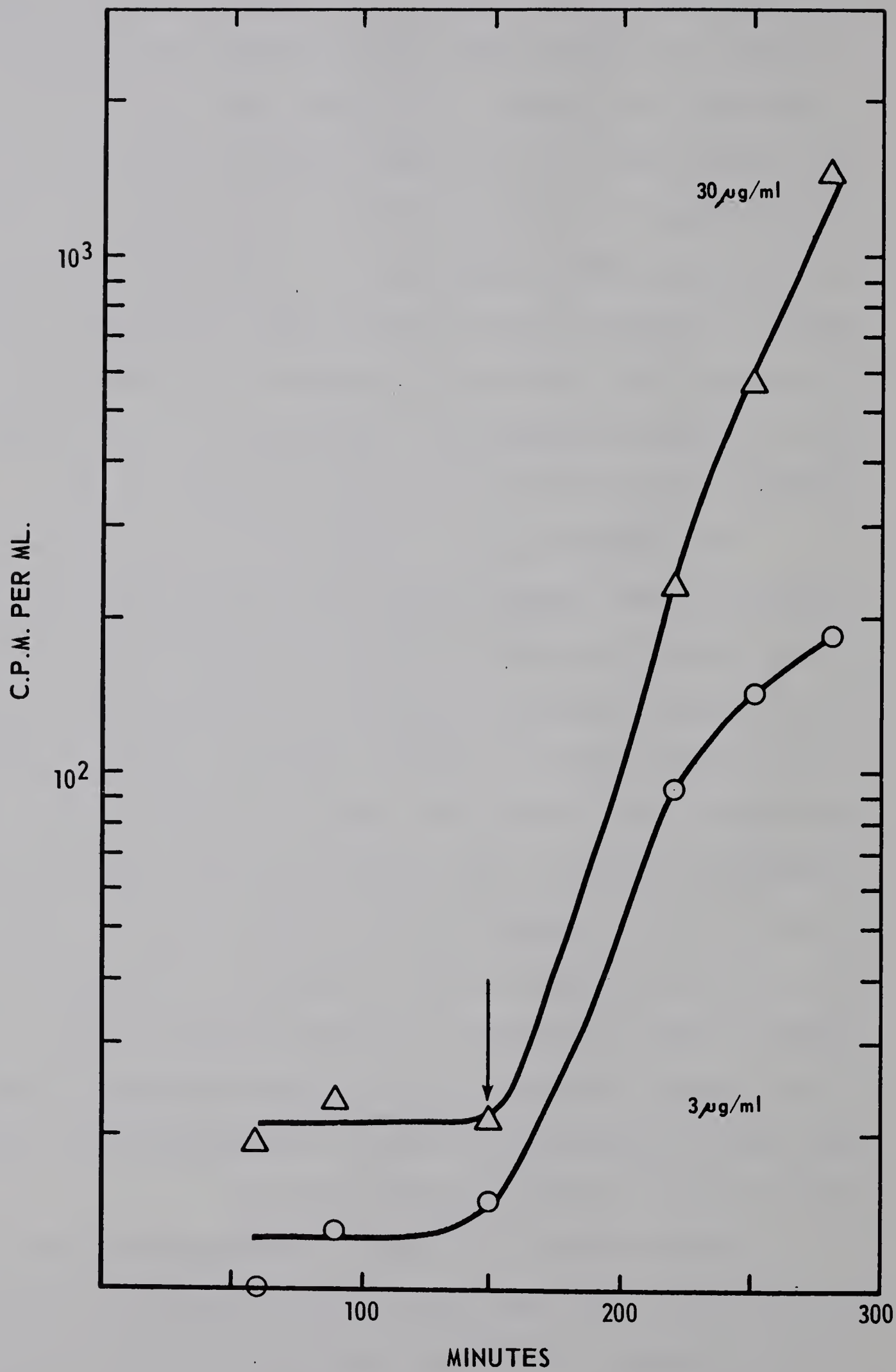


FIGURE 10

THYMINE UPTAKE IN E. COLI B-1

An overnight culture of E. coli B-1 grown on minimal medium supplemented with 4 $\mu\text{g/ml}$ thymine at 30°C was harvested by centrifugation, washed once with medium and resuspended in mineral salts medium supplemented with 30 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$ thymine of specific activity, 2600 c.p.m./ μg and 11,000 c.p.m./ μg respectively. Uptake of radioactivity into the acid-insoluble fraction was followed for 150 min. at 30°C and at this time the temperature of the cultures was increased to 38°C (Arrow). Uptake of radioactivity was followed for an additional 90 minutes.

Conversion of Thymine to Thymidylate - The data accumulated in the mitomycin-C experiments seemed to support the concept that dTMP was the first product accumulated in cells exposed to ^{14}C -thymine, since thymidine could not be detected in the acid-soluble pool of such cells. If thymine were to be converted directly to thymidylate, the reaction would involve transfer of a deoxyribose-5-phosphate group to thymine. The analogous reaction which occurs in ribonucleotide metabolism -- and which is the only mechanism by which E. coli can utilize exogenous purines for growth -- involves the enzyme nucleotide pyrophosphorylase which mediates the reaction of 5-phosphoribosyl 1-pyrophosphate with a base (Buchanan and Hartman, 1959):



It is likely that a 1-pyrophosphate ester of deoxyribose 5-phosphate would be very unstable, but since the 1-phosphate exists, the possible function of 5-phosphodeoxyribosyl 1-pyrophosphate cannot be ignored in the absence of other satisfactory mechanisms. However, the results of experiments to be described below, seem to rule out the participation of this compound in the conversion of thymidine to thymidylate.

Another possible reaction leading to thymidylate formation from thymine would be the direct transfer of deoxyribose 5-phosphate from a deoxyribonucleoside 5'-phosphate to thymine. This reaction is analogous to the transfer of deoxyribose from one pyrimidine to another, a reaction which occurs in a number of systems, mammalian as well as bacterial.

Following this rationale, the formation of thymidylate should occur in a cell-extract incubated with thymine and a suitable deoxy-

ribonucleotide. Accordingly, preliminary experiments were run employing various deoxynucleotides as sources of deoxyribose 5-phosphate. Conversion of thymine to thymidylate was conveniently followed by measuring formation of negatively-charged products retained on DEAE cellulose discs (Materials and Methods). Using dCMP it was found that radioactivity was retained on the disc and that the amount of radioactivity increased linearly with time (Figure 11). Paper chromatography of the deproteinized reaction mixture in solvent A revealed the presence of dTMP and a trace of thymidine. Presumably the thymidine arises as a result of a phosphatase, since there appears to be a phosphatase in E. coli specific for dTMP and dUMP (Wickland and Razzell, unpublished observations). Formation of dTMP was dependent on the presence of a deoxynucleotide, of which dCMP was the best (Table III). Figure 12 illustrates the effects of combinations of deoxynucleoside 5'-phosphates with dCMP on the rate of formation of dTMP. It is obvious that the addition of these compounds results in a marked stimulation of the reaction and that dUMP exhibits the greatest effect. It was found that heating of the extract for 5 minutes at 55°C resulted in a two-fold increase in activity. At the same time a purification resulted, since about 25% of the protein could be removed by centrifugation after this treatment. Although heating did not increase subsequent stability, this treatment was routinely performed on all enzyme preparations prepared later.

The addition of deoxynucleoside triphosphates to the reaction mixture stimulated the rate considerably with dCTP being the most effective (Table IV). The presence of all three nucleotides (dCTP, dATP, dGTP) appeared to result in a cumulative effect since the reaction

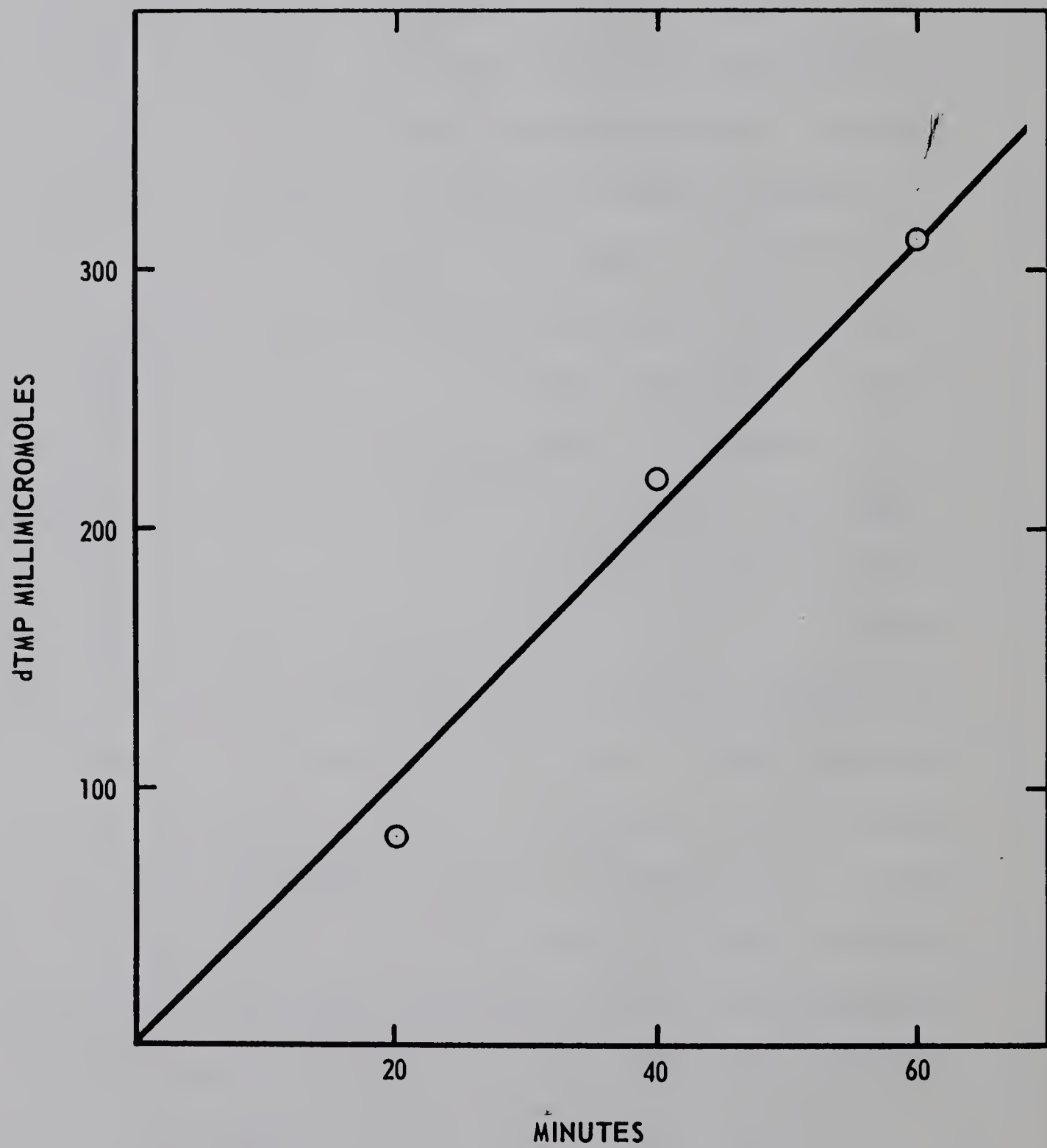


FIGURE 11

THYMIDYLATE FORMATION IN A CELL-EXTRACT

OF E. COLI 5275

The complete system (50 μ l) contained 100 m μ moles dCMP, 30 m μ moles 14 C-thymine (specific activity 6.1 μ C (1.5×10^6 c.p.m.) per μ mole) in 0.05M Tris-malate buffer, pH 7.1, and 25 μ l of cell extract. 15 μ l aliquots were removed onto the DEAE discs and dTMP formation was determined as described in Materials and Methods.

TABLE III

EFFECT OF VARIOUS DEOXYNUCLEOSIDE 5'-PHOSPHATES
ON THE RATE OF FORMATION OF dTMP FROM THYMINE

Additions	dTMP Formed
	c.p.m.
dCMP	2400
dGMP	260
dUMP	350
dAMP	235

The complete system and procedure used was the same as that described in Figure 11. The deoxynucleosides were present at a concentration of 2 μ moles/ml.

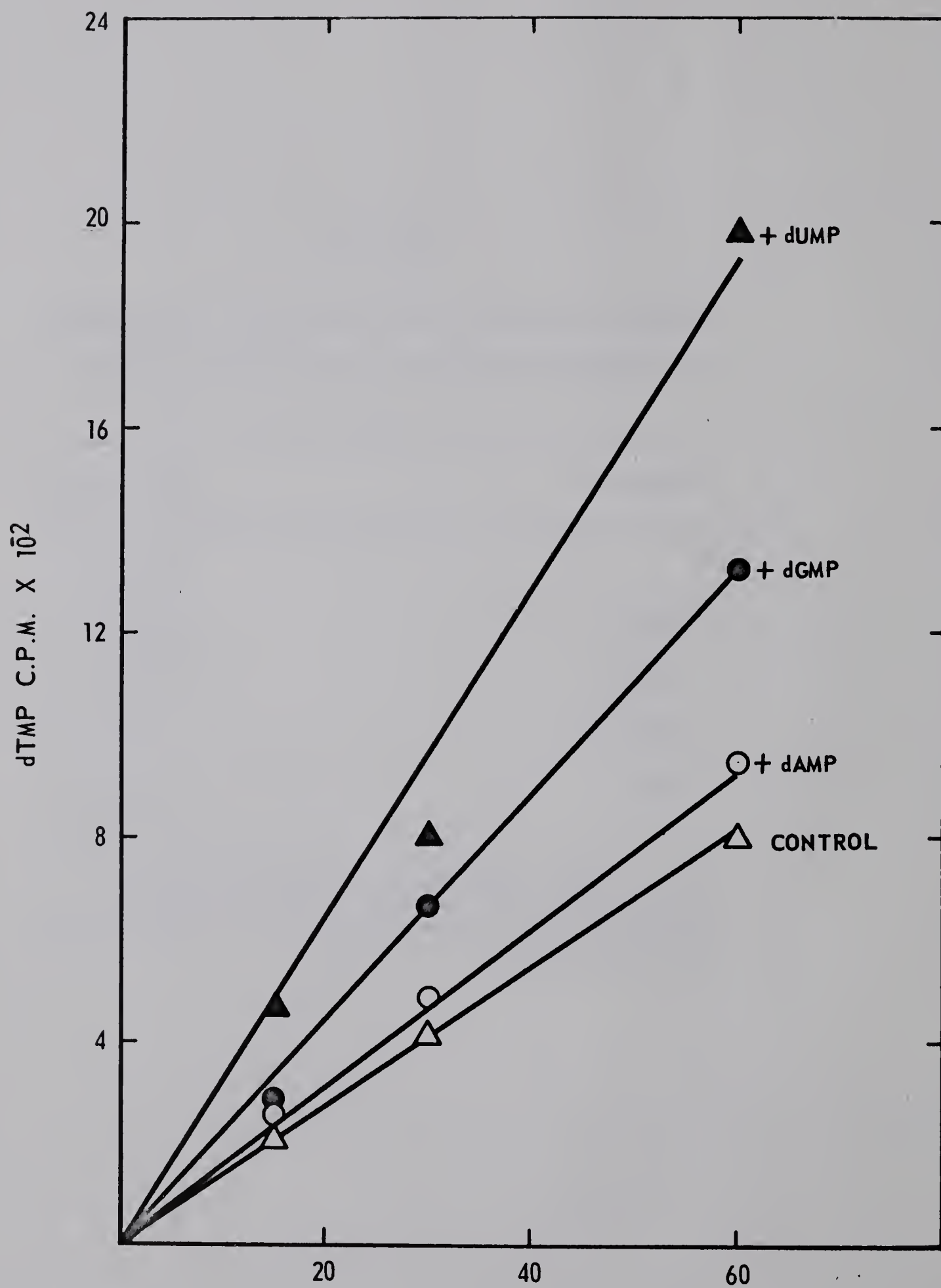


FIGURE 12

EFFECT OF VARIOUS DEOXYNUCLEOSIDE PHOSPHATES ON RATE
OF dTMP FORMATION FROM dCMP

The assay mixture and procedure of Figure 11 was used except that addition of 100 μ moles of various deoxynucleoside phosphates as well as dCMP was made where indicated.

TABLE IV

EFFECT OF DEOXYNUCLEOSIDE TRIPHOSPHATES
ON RATE OF dTMP FORMATION

Additions	dTMP Formed c.p.m.
Control	2000
dATP	5400
dGTP	5400
dCTP	6000
1/3 of all three	8700

The complete system (50 μ l) contained 100 μ moles dCMP, 100 μ moles dUMP, 30 μ moles 14 C-thymine (see Figure 11 for sp. act.), in 0.05M Tris-malate buffer, pH 7.1, and 25 μ l of cell extract. 50 μ moles of the various nucleoside triphosphates were added when indicated. Determination of dTMP formation was the same as described in Figure 11.

rate was greater than that produced by any of the triphosphates when present singly, even though the total concentration of the deoxy-nucleotides was the same in both cases. Further attempts to manipulate this system by the addition of inorganic phosphate or pyrophosphate to dialyzed cell extracts, which should stimulate the rate of dTMP formation if an intermediate phosphate ester was involved, were unsuccessful. In fact, these compounds exhibited strong inhibitory effects above 0.08 M.

Activation and inhibition by end-products in metabolic pathways is very common, and the action of the nucleoside triphosphates illustrated here may be an example of an allosteric interaction. The results of these activating effects seemed consistent with the requirements of any system for converting exogenous thymine to TMP, since an auxotroph dependent on such a reaction would be expected to accumulate some of the DNA precursors dGTP, dCTP and dATP. This accumulation in turn could serve to activate the system supplying dTTP in an effort to relieve the block in DNA synthesis. This then may represent another example of the delicate controls over the metabolic machinery of the cell.

Conversely, when dTMP synthesis was examined in the presence of dTTP (the ultimate end-product of this pathway and a compound which might be expected to inhibit this reaction) it was indeed found that inhibition resulted (10^{-4} M dTTP produced 50% inhibition in the absence of dCTP).

Effect of pH - The optimum pH for the initial reaction rate was determined by assaying dTMP formation in a Tris-malate buffer system at pH values between pH 5.5 and pH 8.5 using a heated extract as a source of enzyme. The substrate and extract were brought to the desired

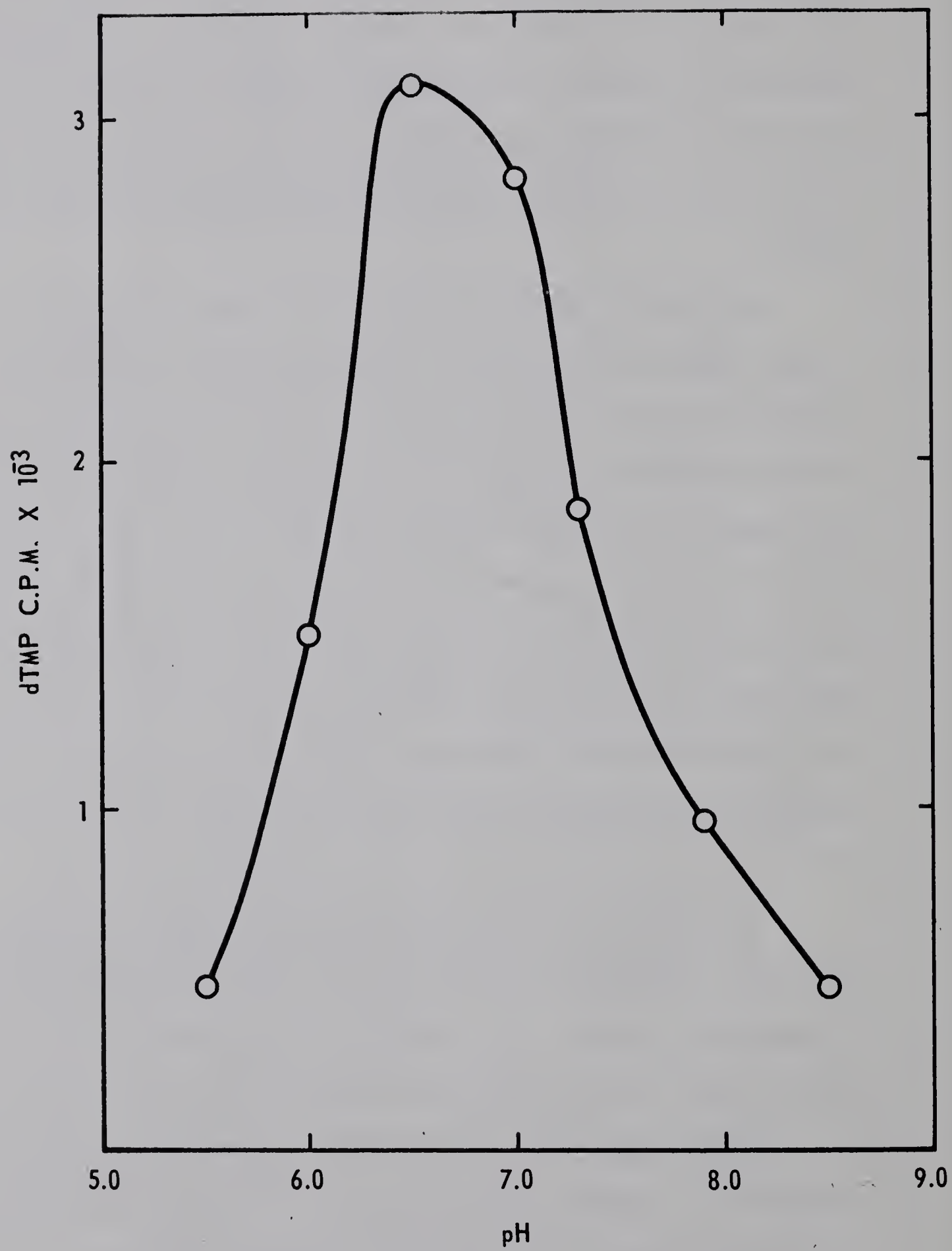


FIGURE 13

THE EFFECT OF pH ON dTMP FORMATION

The assay mixture was the same as described in Table IV.
dTMP formation determined as described in Figure 11.

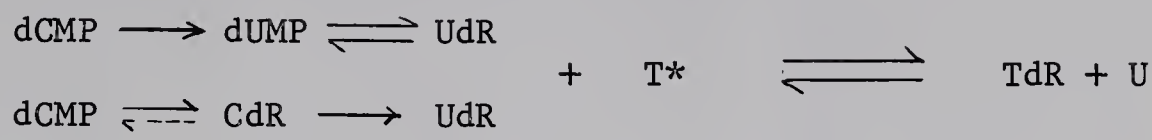
pH before the reaction was initiated. The results are shown in Figure 13. The optimum for the initial reaction rate is approximately pH 6.5.

Attempts to Purify the Enzyme Activity - Attempts to purify or study the activity from extracts of strain 5275 were difficult because of the extreme instability of the enzyme. Upon overnight storage at 5°C about 40% of the original activity was lost. The activity was stable to freezing, but immediately upon thawing, inactivation began. Dithiothreitol (DTT) (0.005 M) showed slight stabilizing effects but did not prevent rapid loss of activity. Dialysis against Tris-malate buffer containing 0.005 M DTT resulted in complete loss of activity after 12 hours. Since the activity from strain 5275 was so unstable, extracts from strain 15 TAU⁻ were surveyed for ability to synthesize dTMP after heat treatment and found to possess an enzyme of about the same specific activity as that of 5275. Little activity was detected without prior heat treatment. These preparations were much more stable than those of 5275 and could be stored, with little loss of activity, for several weeks; furthermore, they could be dialyzed overnight with no loss of activity.

Attempts to purify this activity from the 15 TAU⁻ extract met with failure as well, since the enzyme was lost during the manipulations. The following procedures were tried: alumina gel adsorption, ammonium sulfate fractionation, isoelectric precipitation, DEAE-cellulose and DEAE-Sephadex chromatography, and Sephadex G-200 chromatography. Although activity was recovered from the Sephadex G-200 column the specific activity was no higher and the recovery very low.

Although this crude system will form dTMP from thymine, a number of known reactions could be invoked to explain the results. The possible

reactions by which dCMP could be converted to a deoxyribose donor are shown below.



Route II

Following this line of reasoning, dCTP could conceivably phosphorylate TdR, formed from the reaction between thymine and deoxyuridine. If this were true, dCMP deaminase forming dUMP should make it the sole essential mononucleotide. Table III shows that dUMP is not as effective as dCMP in the reaction. Also low levels of CdR and UdR, expected from the dephosphorylation of dCMP and rapid deamination of deoxycytidine, are no better reactants with thymine for dTMP formation than is dCMP. A further obstacle to the acceptance of this variation of route II is the observation that there is a greater than six-fold difference in rate of dTMP formation from thymine in the presence of dCMP and dCTP when dialyzed extracts from thymineless and wild-type cells are compared in this system (Figure 14). The specific activities of the extracts from B, K12, and 15 TAU⁻ are 3.3, 7.6, and 30 $\mu\text{moles/hr./mg protein}$, respectively. No combination of reactions discussed so far provides a system which would differ between prototrophs and auxotrophs.

If the reaction was proceeding through either of the routes postulated on page 36, ³²P-labelled dCMP would give rise to label in dTMP. Deoxycytidine 5'-phosphate labelled with ³²P was therefore synthesized to test these hypotheses. Chromatography of a reaction mixture containing dCTP, dCM³²P and thymine after 30 minutes incubation did not reveal the presence of dTM³²P. Unexpectedly, however, it was found that most of the dCMP had been converted to a compound with an

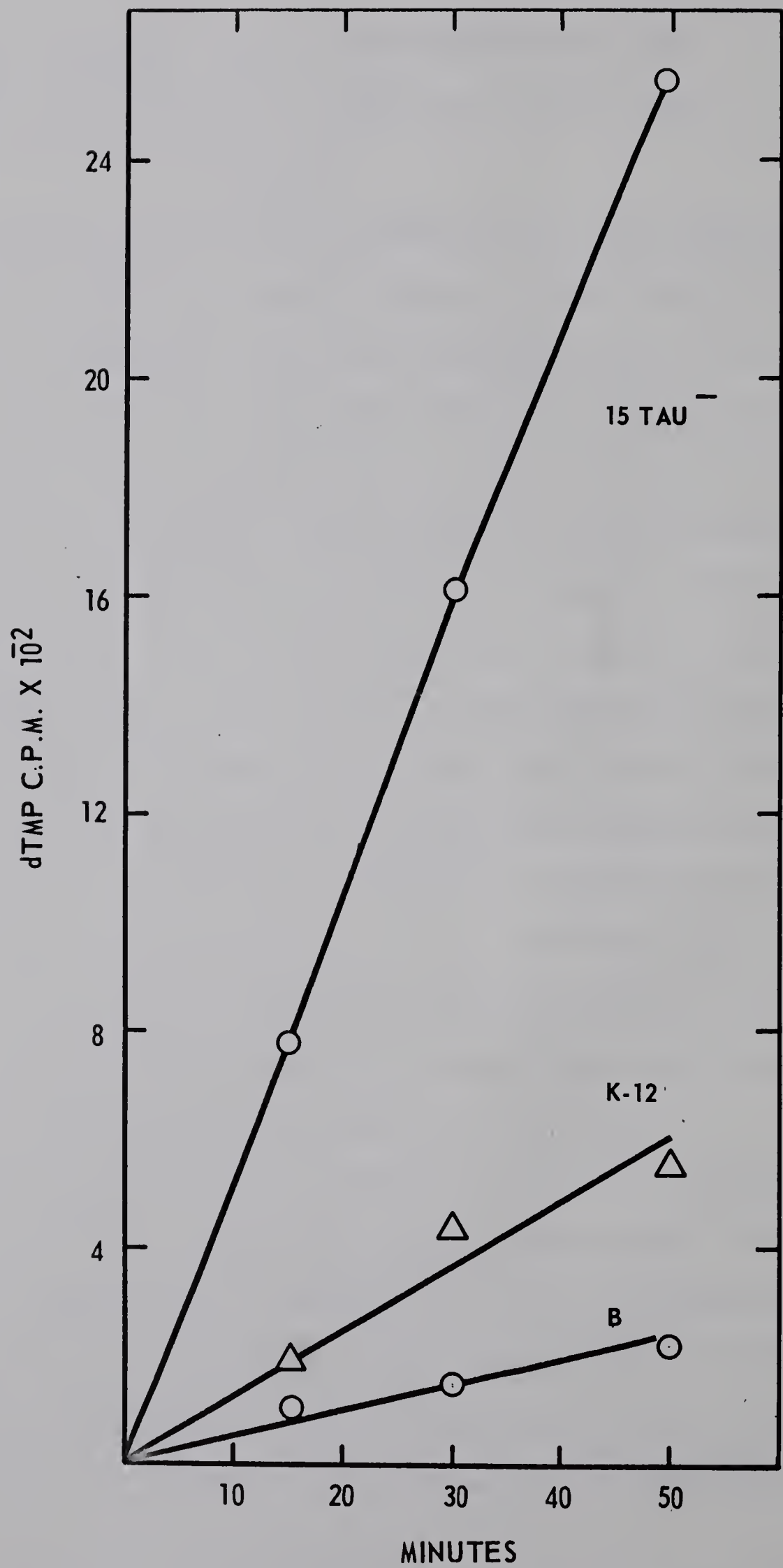


FIGURE 14

dTMP FORMATION BY THREE STRAINS

OF E. COLI

The assay described in Table IV was used. Heated extracts which had been dialyzed for 6 hours were used as sources of enzyme.

R_F similar to dUMP. Further experiments revealed that indeed dCMP was deaminated to dUMP. The occurrence of this deaminase reaction presented difficulties which could not be overcome, so this approach was abandoned. The presence of dCMP deaminase was surprising, since several workers have reported that E. coli does not possess this enzyme except when infected with T-even bacteriophage (Flaks and Cohen, 1959; Keck et al., 1960; Fleming and Bessman, 1967).

DISCUSSION

Possession by *E. coli* of thymidylate synthetase and ability to incorporate exogenous thymine are apparently independent traits and tend to be mutually exclusive: bacteria which possess good thymidylate synthetase activity and therefore are thymine-independent, incorporate thymine poorly. The data presented here, coupled with the work of Kammen (1964), show that permeability restrictions alone do not account for this difference; nor does degradation of thymine occur in growing cultures of wild-type or mutant *E. coli*.

In the present study the ability of mitomycin C to increase the intracellular pool of thymine metabolites has been demonstrated. The reactions leading to the formation of phosphorylated thymine derivatives do not appear to be affected by this drug. The dTMP which is formed must arise as a result of synthesis and not from DNA degradation, since it has been shown that mitomycin C does not cause DNA breakdown in *E. coli* before 60 minutes after addition (Sekiguchi and Takagi, 1960). These workers also found that deoxyribosidic compounds (measured by bacteriological assay) increased after addition of mitomycin C. The later DNA breakdown described by this group probably arises as a result of induction of temperate phage (Suzuki and Kilgore, 1967).

A comparison of ^{14}C -thymine incorporation in two thymineless strains exposed to mitomycin C (Figures 4 and 7) revealed a large difference in the labelling patterns. DNA synthesis was effectively blocked in strain 5275, yet in 15 TAU⁻ inhibition at first was marginal and later became non-existent. Similarly the acid-soluble pools of the two strains were markedly different, the pool in 5275 increased extensively while in 15 TAU⁻ little increase was evident. This effect,

however, can probably be explained by the much shorter induction period of the prophages present in strain 15 TAU⁻. Mitomycin C does not inhibit phage DNA synthesis (Pricer and Weisbach, 1964), therefore the resumption of DNA synthesis in strain 15 TAU⁻ (increase in acid-insoluble radioactivity) represents phage DNA synthesis. It is unfortunate that these phages are induced, since the procedure would otherwise offer a relatively simple method of obtaining labelled thymine nucleotides if the incubation period could be prolonged.

The absence of thymidine from the acid-soluble pool of cells treated with mitomycin C was surprising, since thymidine might be expected to be an intermediate on the pathway from thymine to thymidylate. This observation may be explained in two ways: (a) thymidine is not an intermediate on the pathway to dTMP, or (b) the rate-limiting reaction leading to dTMP formation is the synthesis of thymidine. The experiments employing 2,4-dinitrophenol however, did not produce an accumulation of thymidine as would be expected if the second hypothesis were true, although the possibility of an energy-dependent permeation mechanism could also account for this finding. The evidence for the first hypothesis is not conclusive, but later experiments with cell-free extracts also showed that thymidine does not appear as an intermediate between thymine and TMP.

Equally unexpected as the absence of thymidine in mitomycin treated cells was the presence of the two unknown compounds (Figure 8). Although not extensively examined, these compounds appeared to be ribosides rather than deoxyribosides. One of them is probably thymine riboside, a compound which is formed by E. coli extracts (Mantsavinos and Zamenhof, 1961) and which may account for the "uridine inhibition" effect mentioned in the introduction.

Several considerations led to an examination of cell-extracts for thymidylate formation in the presence of thymine and deoxynucleoside phosphate: first, the absence of thymidine from pools of cells grown in the presence of mitomycin C; second, the exclusion of exogenous thymine from the DNA of prototrophs; third, the observation of Goulian and Beck (1966) that the deoxynucleotide, dCMP, accumulates in the pool of cells that are starved for thymine; fourth, the finding of Kammen (1967) that thymidine phosphorylase, a key enzyme of route II is located on the surface of E. coli, a particularly unlikely location for a biosynthetic enzyme since its substrate, deoxyribose 1-phosphate, would be formed intracellularly and might not easily reach the enzyme across the cell membrane.

The formation of thymidylate by extracts of thy⁻ mutants in the presence of dCMP and thymine was indeed found. Other deoxynucleotides such as dGMP, dAMP and dUMP were much less effective than dCMP. The effective participation of dCMP in the formation of dTMP correlates well with dCMP accumulation during thymine starvation.

The activation of dTMP synthesis by various deoxynucleoside mono and triphosphates and inhibition by dTTP can be considered to represent an allosteric interaction, a common phenomenon in biosynthetic pathways. This aspect of the reaction has been discussed in the Results.

The unexpected presence of dCMP deaminase in the cell-free extracts of E. coli was unfortunate, since it obscured any definitive conclusion regarding deoxyribose 5-phosphate transfer from dCMP to thymine. It should be possible, however, to remove this enzyme from extracts of E. coli and thereby prevent dCMP degradation during experiments with ³²P labelled dCMP. The detection of the deaminase in extracts

of uninfected E. coli seems to shed some doubt on the claims of many workers that this enzyme is phage specific (i.e. specified by a phage structural gene) and is not present in uninfected E. coli (Maley et al., 1967 ; Fleming and Bessman, 1967; Flaks and Cohen, 1959; Keck et al., 1960). Its presence in E. coli 15 TAU⁻ described in this thesis may mean that the phage simply induces the host cell to produce more of this enzyme. Isolation of the responsible enzyme would permit a comparison of its structure with that of the "phage-induced" protein.

The inability of wild-type extracts to incorporate significant amounts of exogenous thymine may be explained by the much lower specific activity of the dCMP-dependent synthetic reaction in wild-type cells. Although the specific activity (30 μ moles/hr/mg protein) of the synthetic reaction in thy⁻ cells is very low when compared to some enzymes of E. coli, this activity is higher than that of thymidylate synthetase (15 μ moles/hr/mg protein) possessed by the wild-type strain (Harrison, 1965). Since the thymidylate synthetase is sufficient for normal growth and DNA synthesis of prototrophic E. coli it follows that the activity of the reaction described here is more than adequate to supply the demands for dTTP in DNA synthesis.

The isolation of additional strains of thy⁻ mutants was undertaken to provide a more diverse selection of mutants. The aminopterin-derived auxotrophs represent a distinctive stable and uniform class of thymineless bacteria. All those isolated in this investigation required at least 14 μ g/ml of thymine for growth in contrast to strains 15 TAU⁻ and 5275. The mutants also retain the poor thymine incorporation characteristic of the wild-type at low thymine concentration. From these primary isolates, strains with more efficient thymine incorporation

were selected. Presumably these low thymine-requiring strains are either mutant in the same gene as the low thymine requiring strains 15 TAU⁻ and 5275 or they arose as a result of a mutational event in a second yet unidentified gene involved in thymine metabolism.

It is of interest that strain B appears to give rise to temperature-sensitive thymineless mutants much more readily than does strain K12. Of five thymineless mutants derived from strain B, two were temperature sensitive, whereas none of the eight mutants derived from strain K12 were of this type. The temperature sensitivity of these mutants is presumably due to production of a temperature-sensitive thymidylate synthetase protein. These mutants map at a single site within the thymidylate synthetase structural gene. This locus appears to represent a "hot spot", since of 150 thy⁻ mutants isolated by Alikhanian et al. (1966), 62 were located at this site, and of these, all were temperature-sensitive. Although E. coli appears to have only one gene (structural gene for thymidylate synthetase) genetic studies with B. subtilis indicate that this organism possesses two genes for endogenous synthesis of thymidylate (Wilson et al., 1966).

The examination of thymine uptake in the temperature-sensitive strain B-1 presented a striking illustration that although these organisms possess the ability to incorporate thymine, they do not do so unless their endogenous supply of thymidylate is blocked, in this case by an increase in temperature. This experiment does not provide information whether protein synthesis (i.e. derepression) is necessary during the transition period from wild-type to mutant phenotype or whether this change comes about simply as a result of a modification in the level of some metabolite leading to either an allosteric interaction

or perhaps providing a source of deoxyribose. If the synthesis of protein were required for thymine incorporation then the addition of a drug such as chloramphenicol or puromycin which blocks protein synthesis would be expected to prevent thymine incorporation by the cells when the temperature was increased.

Up to this time we have been emphasizing the importance of conversion of thymine to dTMP through the mediation of dCMP. Breitman and Bradford (1967, 1968), however, claim that route II functions biosynthetically in thymineless cells and that deoxyribose 1-phosphate accumulates because these cells lack deoxyriboaldolase or deoxyribomutase (see pathway page 8), enzymes which degrade this compound. These workers have advanced this hypothesis on the basis of observations on two thy⁻ mutants, one which lacks the aldolase and another which lacks the mutase. Their data supporting this explanation is based only on experiments with two strains. Further confirmation will have to await study of additional strains. In addition, Alikhanian et al. (1966) demonstrated that deoxyribosides will inhibit the growth of some strains of E. coli. Breitman and Bradford (1967) interpret this inhibition in their aldolaseless strain as due to a toxic accumulation of deoxyribose phosphates which cannot be metabolized or excreted. Since Kammen (1967) has shown that thymidine phosphorylase is located on the surface of E. coli it is difficult to envision the interaction of this enzyme with its substrate deoxyribose 1-phosphate, a necessary interaction in the hypothesis of Breitman and Bradford.

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